The Journal of Laboratory and Clinical Medicine

VOL. 50 NOVEMBER, 1957 No. 5

CLINICAL AND EXPERIMENTAL

SITES OF ABSORPTION OF VITAMIN B_{12}

YALE CITRIN, M.D., CARLO DEROSA, A.B.,
AND JAMES A. HALSTED, M.D.
SYRACUSE, N. Y.

ALTHOUGH a very small proportion of extremely large doses (500 to 2,000 μg) of vitamin B_{12} can be absorbed, presumably by diffusion, from most mucosal surfaces, the sites of absorption of the much smaller amounts normally available in the diet have not been defined. In the present study, the capability of various segments of the intestine to absorb vitamin B_{12} was assessed by means of the urinary excretion test after Co^{60}-B_{12} was delivered through an intestinal tube.

The absorption of vitamin B_{12} may be determined by 3 general methods: (1) demonstration of a hematologic response in a patient with pernicious anemia in relapse; (2) demonstration of a rise in serum B_{12}, or an increased urinary excretion, as measured by microbiologic assay; (3) measurement of radioactivity involving stools, urine, or serum, or external monitoring of the liver after administration of labeled vitamin B_{12}. Using 100 μg doses, Monto and Rebuck[1] have demonstrated absorption from the nasal mucosa. Ross and his associates[2] and Reisner and co-workers[3] have shown that some absorption of vitamin B_{12} may occur from either the oral or the rectal route, when doses of 500 μg or more are given to subjects lacking endogenous intrinsic factor. Using a test dose of 0.5 μg, McIntyre and his associates[4] found significant

From the Veterans Administration Hospital, and the Department of Medicine, State University of New York, College of Medicine in Syracuse.

Presented at the annual meeting of the American Society for Experimental Pathology, Chicago, Ill., April 15-19, 1957.

Supported in part by grants from Merck & Company, Inc., Rahway, N. J., and Organon, Inc., Orange, N. J.

Received for publication April 26, 1957.

urinary recovery of cobalt^{60}-labeled vitamin B_{12} after it was introduced directly into the jejunum of 2 patients with jejunostomies. Using timed-release capsules containing 4 μg test doses, Johnson and Berger[5] have likewise demonstrated significant absorption from the lower small intestine of normal subjects.

METHODS

Twenty-one hospitalized subjects were studied as the "normal" group. None had achlorhydria, anemia, intestinal malabsorption, or neurologic or renal disease. Seven hospitalized subjects with Addison's pernicious anemia in remission were similarly studied. None of the patients was seriously ill.

The following modification of the urinary excretion test[6-8] was employed. A test dose of 0.5 μg of Co^{60}-B_{12}* containing 0.25 μc of radioactivity was given to a fasting patient either by mouth or through a tube. Simultaneously, 1,000 μg of nonradioactive vitamin B_{12} was given intramuscularly. All urine was collected for 24 hours, at which time a second intramuscular injection of 1,000 μg was given, again followed by collection of the urine for the next 24 hours. Four hundred milliliters of each sample was counted in a scintillation counter with special shield. Urinary excretion of Co^{60}-B_{12} was expressed as percentage of the administered dose. The range of excretion in normal patients for the two 24-hour collections in our laboratory was 7 to 43 per cent. A third 24-hour urinary collection after 1,000 μg of vitamin B_{12} was given intramuscularly at 48 hours has failed to yield significant urinary radioactivity. In the patients with pernicious anemia, neutralized human gastric juice pooled from 3 or 4 normal individuals was added to the test doses indicated in Table III. Bifacton sec (Organon), an extract of hog's stomach mucosa, which has intrinsic factor activity but contains no vitamin B_{12},† was added to the test dose in other tests.

Eighteen tests were performed by the rectal route of administration in 14 subjects without pernicious anemia. From 0.5 to 3.0 μg of Co^{60}-B_{12} containing from 0.25 to 3.2 μc of radioactivity was instilled through a rectal tube, passed to a level of at least 15 cm. in a patient in the knee-chest position. The subject remained in this position for 15 minutes, and then in the prone Trendelenburg position for one hour. No subject had a bowel movement for at least 4 hours thereafter. The "flushing" doses, of 1,000 μg of nonradioactive vitamin B_{12} were given parenterally at 2 and 26 hours after the instillation. The collection of urine for 2 consecutive 24-hour periods was accomplished as described above. Gastric juice in one test and Bifacton sec in 5 tests was mixed with the test dose. One subject was given twelve grams of neomycin on the day preceding the test. Three subjects were given 250 mg. of tetracycline orally every 6 hours for 4 days before their tests. Tetracycline was likewise given to two patients for 6 and 8 days, respectively, at which time the test dose was administered with intrinsic factor. One dose was delivered to the transverse colon, and one to the cecum, through the intestinal tube described below. Intrinsic factor was added to the dose delivered to the transverse colon.

Intestinal intubation was carried out as follows. A modification of the polyvinyl tube described by Blankenhorn and associates[9] was followed down the small intestine under fluoroscopic and roentgenographic control. Using barium sulfate, with contrasting air insufflation, the pylorus, duodenojejunal junction, and ileocecal valve were used as primary landmarks. The mucosal pattern and the general topography involved were considered as secondary landmarks in identifying the location of the tube. Neither the length of the tube passed, nor the time involved in passage was helpful. If reasonable doubt existed as to the localization, the study was discarded. The effect of barium sulfate on the absorption of Co^{60}-B_{12}, when given orally, was tested in two subjects by adding 50 ml. of a 1:1 barium sulfate suspension to the test dose. The oral urinary excretion test as defined above was repeated 5 times at 7-day intervals in 3 subjects without pernicious anemia. One subject had 4 tests.

*Generously supplied by Dr. Nathaniel L. Ritter, Merck & Company, Inc., Rahway, N. J.
†Kindly supplied by Dr. Kenneth W. Thompson of Organon, Inc., Orange, N. J.

RESULTS

The reproducibility of the urinary excretion test is shown in Table I. Although there are too few tests for statistical analysis, a surprising degree of consistency was obtained in the same subject. It is also apparent that barium sulfate had no inhibitory effect on the absorption of vitamin B_{12}.

TABLE I. REPRODUCIBILITY OF A 2-DAY URINARY EXCRETION TEST IN NORMAL SUBJECTS

	PER CENT OF 0.5 μG Co^{60}-B_{12} SUBJECT:			
TEST*	1	2	3	4
1	39.4	30.4	14.0	13.7
2	31.6	28.8	9.9	13.2
3	30.9	29.8	9.7	10.7
4	30.6	27.2	9.2	8.1
5		27.8	9.2†	13.0†

*Tests done at 7-day intervals.
†Fifty milliliters barium sulfate mixed with dose.

TABLE II. URINARY EXCRETION TEST IN NORMAL SUBJECTS

		PER CENT OF 0.5 μG Co^{60}-B_{12} SITE OF DELIVERY OF TEST DOSE:			
SUBJECT	AGE	STOMACH	DUODENUM	JEJUNUM	ILEUM
G. S.	30	17.7	39.5		
G.	49	24.6	15.6		
H.	46	16.4	25.3		
McC.	41	13.6	30.2		
O.*	57	9.2*	14.7		
D.*	58	13.0*	22.2		
W. E.	60	16.1	26.5	22.8	16.5
D. P.	39			22.3	
D. M. S.	42				23.2
H. B.	66	21.1			0†
E. J. R.	33	21.5		12.3	
R. W.	26				4.7†
R. H. B.	42				18.4†

*Test No. 5 in Subjects 3 and 4 in Table 1.
†Ileocecal valve visualized.

TABLE III. URINARY EXCRETION TEST IN SUBJECTS WITH PERNICIOUS ANEMIA

		PER CENT OF 0.5 μG Co^{60}-B_{12} SITE OF DELIVERY OF TEST DOSE:				
SUBJECT	AGE	STOMACH (TEST DOSE ALONE)	STOMACH (TEST DOSE PLUS I.F.*)	DUODENUM (TEST DOSE PLUS I.F.)	ILEUM (TEST DOSE ALONE)	ILEUM (TEST DOSE PLUS I.F.*)
A. E.	78	0				3.6
G. N. N.	58	2.2	17.6			13.0
P.	58	0	6.8			5.7
M. A.	71	0	13.0		0	
Y.	72	0	5.3			4.9
W.	66	0	9.7b	6.9b	0	
		3.8	12.6		0	15.3
B.	79	0				22.3

*I.F. = gastric juice, 25 to 100 ml. (except b = Bifacton sec, 30 mg.).

Table II shows the results of delivery of vitamin B_{12} by intestinal intubation in normal subjects. As can be seen, the recovery following duodenal instillation was significantly greater than in the case of the oral route in 6

of 7 subjects. These differences are especially striking when they are compared to the maximum range of variation found in the reproducibility tests. Significant absorption from the jejunum occurred in 3 subjects and from the ileum in 3 others. Poor absorption from the ileum was noted in 2 additional subjects.

Seven subjects with pernicious anemia were tested with and without intrinsic factor (Table III). Without added intrinsic factor, Co^{60}-B_{12} was not absorbed from the ileum of two patients. With added intrinsic factor, there was significant absorption from the ileum in 6 patients, and from the duodenum in one.

In the 18 tests involving the administration of Co^{60}-B_{12} into the colon, no absorption could be demonstrated in any case. These tests included 6 in which intrinsic factor was added to the test dose, as well as 6 in which antibiotic administration preceded the test dose, given with and without intrinsic factor.

DISCUSSION

By the technique described, rectal instillation probably causes spread of the test dose to at least the level of the splenic flexure. Truelove,[10] by adding contrast material to a rectal drip in a supine patient, has radiographically demonstrated spread of the solution to the splenic flexure and beyond. Forceful injection through a tube in a patient in the knee-chest position followed by the prone Trendelenburg position should, therefore, result in distribution of the test dose at least over the descending colon, and probably to the transverse colon.

It was evident from roentgenograms that the fluid forced under pressure through a narrow bore tube resulted in splattering of the solution over many inches of intestine. Thus it is possible that a portion of the Co^{60}-B_{12} instilled into the terminal ileum might immediately splash into the colon. This might explain in part the low urinary excretion obtained in two subjects, in both of whom the ileocecal valve was visualized within a foot of the tube tip.

The results indicate that in the normal subject vitamin B_{12} in doses of 0.5 μg can be absorbed from the entire small intestine. Inasmuch as intrinsic factor is secreted only in the stomach[11] it is presumed that secretion from that site is available down to the terminal ileum as a result of peristaltic action. Absorption of Co^{60}-B_{12} from the ileum of subjects with pernicious anemia occurred only when intrinsic factor was added to the test dose. Therefore, the ileum is capable of absorbing vitamin B_{12} but only when intrinsic factor is present.

It has been suggested that the process of absorption of vitamin B_{12} normally takes place relatively high in the small intestine.[12] The demonstration that the entire small intestine is capable of absorbing vitamin B_{12} does not invalidate this opinion, for under the normal circumstances of diet and health, vitamin B_{12} is probably absorbed primarily in the more proximal regions of the intestine. The variability of absorption observed from the ileum of normal subjects would appear to be a reflection of either the inconstant

availability of endogenous intrinsic factor in the ileum, as a result of destruction, utilization, or dilution in its transport from the stomach, or the splashing effect described previously with an immediate loss into the colon of part of the test dose.

The inability of the colon to absorb vitamin B_{12}, despite exogenous intrinsic factor, suggests that the mucosa of the colon lacks the necessary transport mechanism. The failure of antimicrobial administration to facilitate absorption suggests that bacterial competition or binding in the colon is not the explanation.

The striking enhancement in absorption which occurred in 6 of 7 subjects when the stomach was by-passed by duodenal intubation is of considerable interest. Previous observations have suggested that the stomach may actually play an inhibitory role in absorption. Halsted and associates[11] performed fecal recovery tests in 11 patients with a total gastrectomy after gastric juice was added to the test dose of Co^{60}-B_{12}. In seven, absorption occurred which was much greater than that exhibited either by normal individuals, or by patients with pernicious anemia given gastric juice with the test dose. Furthermore, Johnson and Berger,[5] using timed-release capsules, demonstrated that urinary excretion was greater when Co^{60}-B_{12} was released in the small intestine rather than in the stomach.

The binding of vitamin B_{12} by proteins without intrinsic factor activity,[14] making it unavailable for absorption, is a possible explanation for an inhibitory role of the stomach. However, the effect of pH on intrinsic factor may offer a more likely explanation. As early as 1937, Castle and associates[13] described the importance of pH on intrinsic factor-extrinsic factor mixtures. Beef muscle and gastric juice incubated at pH 2.5 for 12 hours was hematopoietically inert unless partial neutralization to pH 5 was made before administration. More recently, from the same laboratory,[15] experiments using gastrectomized rats have shown that incubation of a mixture of Co^{60}-B_{12} with homogenized rat stomach at an acid pH reduced the absorption of the vitamin. Gräsbeck,[16] studying gastric juice by the electrophoretic method, described a striking loss of the main vitamin B_{12} binding fraction (which he speculates may be intrinsic factor) after incubation of the gastric juice at acid pH for longer than 30 minutes. If digestion of intrinsic factor should occur after it binds B_{12}, a large portion of the B_{12} might become unavailable for absorption. On direct instillation into the more alkaline environment of the duodenum, the B_{12} might be preferentially bound to intrinsic factor that has escaped digestion. Therefore, it would appear that the destructive effect of an acid environment on intrinsic factor may be the result of proteolytic digestion as suggested by Castle and Ham in 1936.[17] This may be a possible explanation for the "inhibitory" effect of the stomach.

CONCLUSIONS

1. In doses of 0.5 μg vitamin B_{12} can be absorbed from the entire small intestine provided intrinsic factor is available.

2. Absorption of vitamin B_{12} in doses of 0.5 to 3.0 μg from the colon could not be demonstrated in 18 tests despite the addition of intrinsic factor to 6 test doses, and the prior administration of neomycin or tetracycline in 6 tests with or without intrinsic factor.

3. As measured by a 2-day urinary excretion test, the degree of absorption of 0.5 μg of vitamin B_{12} given orally at 7-day intervals was fairly constant in a given individual.

4. Absorption after instillation into the duodenum was significantly greater than after oral administration in 6 of 7 normal subjects. This suggests an inhibitory role of the stomach. Possible explanations for this effect are offered.

REFERENCES

1. Monto, R. W., and Rebuck, J. W.: Observations on the Mechanism of Intranasal Absorption of Vitamin B_{12}, Blood **10:** 1151-1155, 1955.
2. Ross, G. I. M., Mollin, D. L., Cox, E. V., and Ungley, C. C.: Hematologic Responses and Concentration of Vitamin B_{12} in Serum and Urine Following Oral Administration of Vitamin B_{12} Without Intrinsic Factor, Blood **9:** 473-488, 1954.
3. Reisner, E. H., Jr., Weiner, L., Schitlone, M. T., and Hench, E. A.: Oral Treatment of Pernicious Anemia With Vitamin B_{12} Without Intrinsic Factor, New England J. Med. **253:** 502-506, 1955.
4. McIntyre, P. A., Sachs, M. V., Krevans, J. R., and Conley, C. C.: Pathogenesis and Treatment of Macrocytic Anemia, A. M. A. Arch. Int. Med. **96:** 541-549, 1956.
5. Johnson, P. C., and Berger, E. S.: Cobalt^{60}-Labeled Vitamin B_{12} Absorption and Excretion Studies Using Timed Release Capsules (Abstract), Clin. Res. Proc. **4:** 234, 1956.
6. Callender, S. T., and Evans, J. R.: The Urinary Excretion of Labelled Vitamin B_{12}, Clin. Sc. **14:** 295-302, 1955.
7. Schilling, R. F.: Intrinsic Factor Studies. II. The Effect of Gastric Juice on the Urinary Excretion of Radioactivity After the Oral Administration of Radioactive Vitamin B_{12}, J. LAB. & CLIN. MED. 42: 860-866, 1953.
8. Schilling, R. F., Clatanoff, D .V., and Korst, D. R.: Intrinsic Factor Studies. III. Further Observations Utilizing the Urinary Radioactivity Test in Subjects With Achlorhydria, Pernicious Anemia, or a Total Gastrectomy, J. LAB. & CLIN. MED. **45:** 926-934, 1955.
9. Blankenhorn, D. H., Hirsch, J., and Ahrens, E. H., Jr.: Transintestinal Intubation: Technic for the Measurement of Gut Length and Physiologic Sampling at Known Foci, Proc. Soc. Exper. Biol & Med. **88:** 356-362, 1955.
10. Truelove, S. C.: Treatment of Ulcerative Colitis With Local Hydrocortisone, Brit. M. J. **2:** 1267-1272, 1956.
11. Halsted, J. A., Gasster, M., and Drenick, E. J.: Absorption of Radioactive Vitamin B_{12} After Total Gastrectomy, New England J. Med. **251:** 161-168, 1954.
12. Castle, W. B.: Development of Knowledge Concerning the Gastric Intrinsic Factor and Its Relation to Pernicious Anemia, New England J. Med. **249:** 603-614, 1953.
13. Castle, W. B., Heath, C. W., Strauss, M. B., and Heinle, R. W.: Observations on the Etiologic Relationship of Achylia Gastrica to Pernicious Anemia. VI. Site of Interaction of Food and Gastric Factors; Failure of in Vivo Incubation to Produce Thermostable Hematopoietic Principle, Am. J. M. Sc. **194:** 618-625, 1937.
14. Bird, O. D., and Hoevet, B.: Vitamin B_{12}-Binding Power of Proteins, J. Biol. Chem. **190:** 181-189, 1951.
15. Nieweg, H. O., Shen, S. C., and Castle, W. B.: Mechanism of Intrinsic Factor Action in the Gastrectomized Rat, Proc. Soc. Exper. Biol. & Med. **94:** 223-230, 1957.
16. Grāsbeck, R.: Studies on the Vitamin B_{12}-Binding Principle and Other Biocoloids of Human Gastric Juice, Acta med. scandinav. (Suppl.) **314:** 1-81, 1956.
17. Castle, W. B., and Ham, T. H.: Observations on the Etiologic Relationship of Achylia Gastrica to Pernicious Anemia. V. Further Evidence for Essential Participation of Intrinsic Factor in Hematopoietic Responses to Mixtures of Beef Muscle and Gastric Juice and to Hog Stomach Mucosa, J. A. M. A. **107:** 1456-1463, 1936.

FOLIC ACID ABSORPTION, EXCRETION, AND LEUKOCYTE CONCENTRATION IN TROPICAL SPRUE

C. E. Butterworth, Jr., Major, MC, USA,* Hyman Nadel, Ph.D.,* Enrique Perez-Santiago, M.D.,** Rafael Santini, Jr., Ph.D., and Frank H. Gardner, M.D.***
San Juan, Puerto Rico

INTRODUCTION

FOLIC acid has been widely used in the treatment of tropical sprue since its effectiveness was first demonstrated in 1945.[1, 2] Girdwood[3] has shown that patients with sprue excrete less folic acid in the urine after an oral dose than after a parenteral dose, suggesting impaired absorption or tissue depletion. Garcia-Lopez and associates[4] have called attention to the nutritional inadequacy of the diet consumed by patients with sprue in Cuba, but they were able to maintain these patients on a deficient diet and still achieve good hematologic responses with folic acid. However, the roles of dietary deficiency and malabsorption of folic acid remain poorly defined as to the etiology of the disease. For example, a malabsorption syndrome has been observed in military personnel in Puerto Rico who had an adequate diet at their disposal[5] and sprue has been observed in prosperous as well as indigent patients. On the other hand, patients with Whipple's disease or nontropical sprue may show malabsorption without the megaloblastic anemia which is so commonly a feature of folic acid deficiency. It is also curious that sprue is not encountered in certain areas of the world, such as Jamaica, where malnutrition and vitamin deficiency are prominent problems. In considering the role of folic acid in the etiology of sprue, then, the following questions may be asked:

1. Is there an impairment of folic acid absorption from the intestinal contents in patients with tropical sprue?
2. Is intestinal malabsorption the cause or the effect of folic acid deficiency?
3. Is there an intrinsic defect in the absorption of folic acid which produces the disease state?
4. Is the requirement for folic acid higher in patients with sprue than in normal persons?
5. Are there naturally occurring folic acid analogs (e.g., of bacterial origin) which interfere with its utilization? These are some of the questions which prompted the following investigations.

From the U. S. Army Tropical Research Medical Laboratory, San Juan, Puerto Rico.
Received for publication May 13, 1957.
*Walter Reed Army Institute of Research; stationed at the U. S. Army Tropical Research Medical Laboratory.
**Bayamon District Hospital, Bayamon, Puerto Rico.
***Peter Bent Brigham Hospital, Boston, Mass.

MATERIAL AND METHODS

Serum and Urine Determinations.—Folic acid was measured by bacteriologic assay using a commercially available medium (Difco), and *Streptococcus faecalis.** Crystalline folic acid was used as the reference standard for the assays and for the oral doses. The dose used for oral administration was 5 mg. of the crystalline material dissolved in 5 ml. of 20 per cent ethanol. For intravenous injection a commercial preparation (Folvite) was prepared so that 10 ml. contained 5 mg. of folic acid. This was confirmed by the assay procedure.

Urine was collected for 5 hours after the administration of a dose. The output was measured and an aliquot was frozen immediately and stored at -20° C. until the folic acid assay could be performed. Storage did not usually exceed 2 to 3 weeks. All subjects were fasting when the dose was administered. In later studies some were allowed to eat a light breakfast 1 hour after dosage since it has been shown that this does not affect the results.[6] Appropriate urine dilutions were prepared and compared with duplicate dilutions of the reference standard. A standard curve was prepared with each group of assays.

Serum determinations were made on samples taken 1, 2, 3, and 4 hours after oral or intravenous administration. Serum dilutions were prepared aseptically and added to sterile media, since it was found that autoclaving the serum mixtures caused protein precipitation which interfered with turbidity readings. Folic acid-free equipment was used throughout in collecting and handling samples.

Leukocyte Assay.—Leukocyte folic acid was measured by a technique similar to that of Swendseid, Bethell, and Bird.[7] For this procedure, 20 ml. of whole blood were collected, heparinized, and mixed in a test tube with 40 ml. of 1 per cent dextran (average molecular weight 193,000) dissolved in 0.85 per cent saline solution. Red cell sedimentation was usually complete in about 2 hours at which time the leukocyte-rich plasma was transferred to a centrifuge tube and spun at 2,000 g for 10 minutes. The supernatant was removed and the remaining cell suspension was centrifuged at 900 g in a Wintrobe hematocrit tube. The height of the leukocyte column was measured and was converted to milliliters by a factor of 0.067 ml. per centimeter determined by calibration of the hematocrit tubes in use. In most cases the red cell volume was less than 4 mm. in height and the platelet layer less than 3 mm. The white cell column varied from 5 to 20 mm. Observations here and in other laboratories indicate that platelets and red cells do not significantly interfere with the results. The concentrated material was diluted 50 times with equal quantities of distilled water and 1 per cent sodium acetate buffer at a pH of 4.5. This was incubated for 18 hours at 37° C. to liberate conjugated folic acid utilizing the naturally occurring conjugase present in leukocytes. The conjugase activity was inactivated by boiling 10 minutes, after which cellular debris was removed by centrifugation. The supernatant was adjusted to a pH of 6.5 to 7.0 with 1 N NaOH and dilutions were prepared for assay as in the other determinations.

Chromatography.—The 24-hour urine collections of 6 patients with tropical sprue in relapse and of 2 in remission were concentrated by evaporation to a volume of about 2 ml. and one dimensional chromatography was carried out in a buffered solution of mono- and disodium phosphate at pH 7.0. Spots representing 0.01 and 0.02 of the 24-hour urine volume were used. Strips were cut in half, longitudinally, and assayed by the bioautographic technique described by Nichol, Zakrzewski, and Welch.[8] Folic acid, aminopterin, *a*-methopterin, and leucovorin were chromatographed in 1 μg quantities as reference standards. Fountain and associates[9] have shown that boiling in vacuo does not inactivate *a*-methopterin and we have found that boiling for 3 hours without vacuum does not inactivate aminopterin.

Subjects.—Patients referred to as having untreated sprue were diagnosed by the clinical findings of diarrhea, glossitis, and weight loss, together with demonstration in the laboratory of megaloblastic changes in the bone marrow and malabsorption of two or more test substances (xylose, vitamin A, butter, dietary fat). This term was used also for patients in relapse with a similar picture. Treated subjects were those who had previously

*The organism used for this study was *S. faecalis* (29-21) kindly provided by Dr. W. N. Pearson, Department of Biochemistry, Vanderbilt University, Nashville, Tenn.

been studied during relapse, or who gave historical evidence of diarrhea, glossitis, anemia, and who responded to specific therapy. Maintenance therapy consisted of one tablet (5 mg.) of folic acid daily. When treated patients were studied the maintenance dose of folic acid was withheld for one or more days prior to administration of the test dose. Control subjects were laboratory technicians, hospital attendants, or hospital patients without hematologic or gastrointestinal disease.

RESULTS

1. *Urinary Excretion.*—The urinary excretion values in 5 hours after the 5 mg. oral or intravenous dose are summarized in Table I. The difference between the folic acid output of controls and untreated subjects is significant at the 1 per cent level. These results compare favorably with those of Swendseid and associates,[10] who found a normal average excretion of 1.5 mg. in 24 hours (30 per cent of the oral dose), and of Girdwood,[3] who considers excretion of less than 1.5 mg. after a 5 mg. parenteral dose indicative of tissue depletion. The present findings differ slightly from Girdwood's in that treated subjects excreted essentially normal amounts of folic acid, whereas his tropical sprue subjects who were described as being in poor condition showed low excretion values even after treatment.

TABLE I. FOLIC ACID ASSAY: MEAN URINARY EXCRETION IN 5 HOURS AFTER A 5 MG. DOSE

		ORAL			INTRAVENOUS		
SUBJECTS	NO. OF ASSAYS	MG.	S.D.	PER CENT OF DOSE	MG.	S.D.	PER CENT OF DOSE
Normal	22	1.31	±0.44	26.0			
	13				2.02	±0.27	40
Treated for sprue	36*	1.21	±0.40	24.0			
	21†				1.97	±0.84	39
Untreated for sprue	10	0.064	±0.071	1.2			
	8				1.39	±0.57	27

*Includes 22 subjects of whom 14 also had a repeat test at a later date.

†Includes 16 subjects of whom 5 also had a repeat test at a later date. In all other cases there was one assay per subject.

TABLE II. FOLIC ACID: MEAN URINARY EXCRETION VALUES IN 5 HOURS FOLLOWING A 5 MG. DOSE

SUBJECTS	ORAL DOSE (PER CENT EXCRETED)	INTRAVENOUS DOSE (PER CENT EXCRETED)
4 untreated patients	0.6	24
3 of original 4 patients after:		
1 to 2 mo. treatment	19.0	29
4 to 5 mo. treatment	- -	32

Serial observations were made on 4 subjects before and during treatment. The results are summarized in Table II. It may be seen that within 4 to 8 weeks of folic acid therapy the urinary excretion of folic acid after a 5 mg. oral dose was about 30 times greater than before therapy. The output after intravenous administration improved slowly but had not reached the normal value after 4 to 5 months of therapy.

2. *Serum Levels.*—The serum concentrations after oral administration are shown graphically in Fig. 1.

A "t" test comparing the 2-hour value of untreated subjects with the normal 2-hour value indicates significance at the 1 per cent level.

Serum concentrations were determined after intravenous administration of the 5 Gm. doses to 2 normal persons, 9 treated and 3 untreated sprue patients (Fig. 2). Over 80 per cent of the injected dose was no longer measurable at the end of one hour. It appears that clearance proceeds at a rather rapid rate in each type of subject. There was no trend in this small group of subjects suggestive of an avidity for folic acid in sprue, comparable to that noted with vitamin C in scurvy. It appears that plasma clearance is essentially the same whether the folic acid enters the urine or tissues. Since the method described measures only free folic acid some of the clearance could be due to binding by plasma protein.

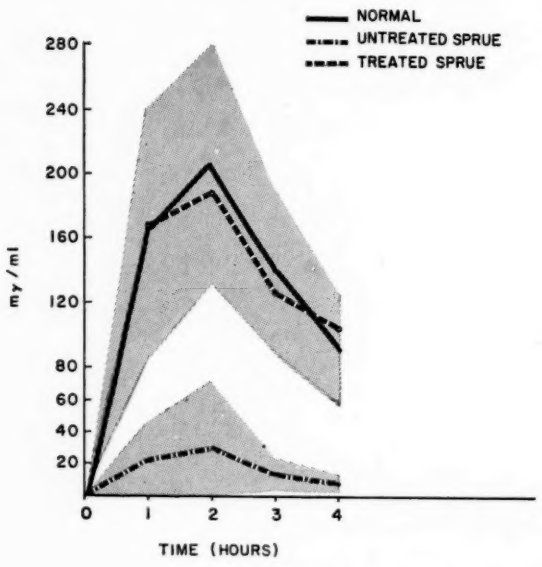

Fig. 1.

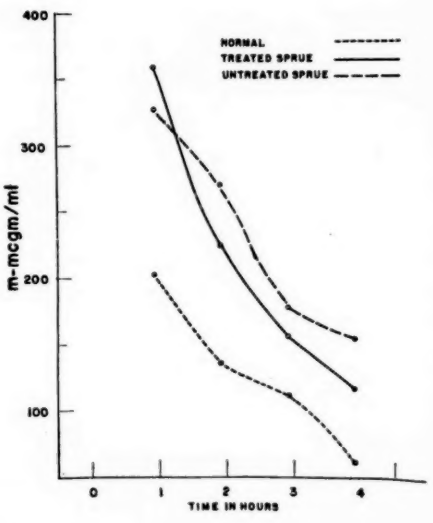

Fig. 2.

Fig. 1.—Mean serum folic acid concentrations at hourly intervals after the oral administration of a 5 mg. folic acid dose to 4 normal subjects, 5 patients with treated sprue, and 6 patients with untreated sprue. The shaded area represents one standard deviation above and below the mean value for normal and untreated subjects. The standard deviation for treated subjects has been omitted for the sake of clarity but is similar to that of the normal group.

Fig. 2.—Mean serum folic acid concentrations at hourly intervals after the intravenous administration of a 5 mg. folic acid dose to 2 normal subjects, 9 patients with treated sprue, and 3 patients with untreated sprue.

3. *Leukocyte Folic Acid.*—The results of leukocyte assay are summarized in Table III. The difference between the untreated sprue group and the normal subjects is significant at the 1 per cent level.

TABLE III

SUBJECTS		WBC FOLIC ACID IN MμG PER ML.		
		RANGE	MEAN	S.D.
Normal	13	46- 97	69.3	15.2
Treated for sprue	6	58-114	81.5	19.2
Untreated for sprue	12	<24- 66	49.1	11.5

4. *Recovery Tests and Chromatography.*—Since the method of urinary assay described does not take into account the possibility that natural antimetabolites are present, recovery experiments were performed in an effort to determine if competition with some inhibitor could explain some of the low levels of urinary folic acid excretion previously noted in untreated subjects. The average amount recovered in 5 normal urines was 115 per cent (range 99 to 126) and in 8 treated patients the recovery was 96 per cent (range 86 to 109). Girdwood[3] has also reported satisfactory recovery of folic acid added to urine. Before untreated subjects were studied it was decided to abandon this method of approach in favor of the more sensitive chromatographic technique.[8]

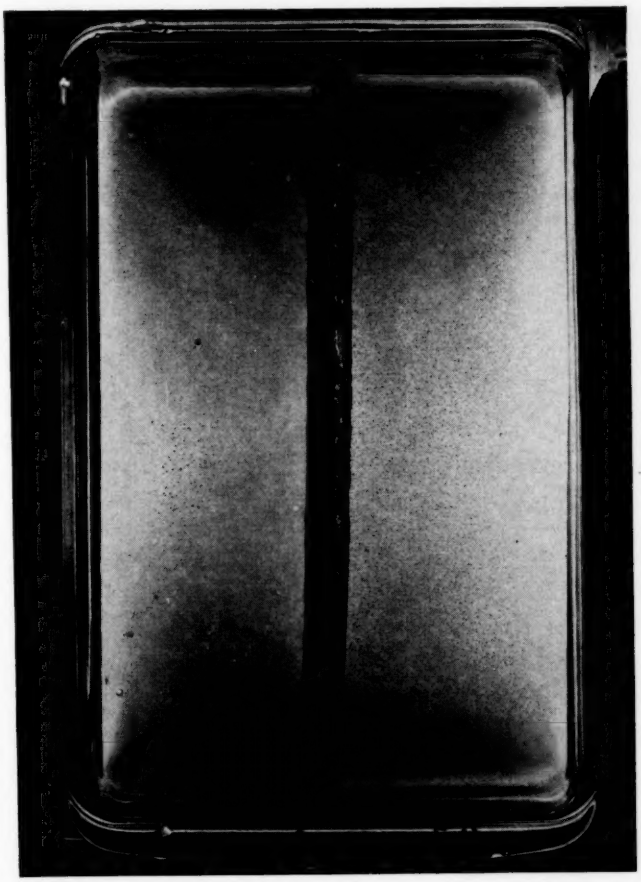

Fig. 3.—Bioautograph of a paper strip chromatogram of aminopterin. The strip was cut in half longitudinally, after which half was applied for 10 minutes to the surface of the folic acid-free medium on the left and the other half to the medium containing folic acid on the right. Growth occurred in the compartment on the left corresponding to the Rf value for folic acid known to be present in aminopterin and inhibition occurred in the compartment on the right at the Rf value for aminopterin.

This method permits detection of certain folic acid inhibitors occurring in the same specimen as folic acid, while the recovery technique might fail to reveal inhibition in the presence of an excess of folic acid. The results of chromatography and bioautographs are depicted in Figs. 3 and 4.

A growth zone was observed in the folic acid free medium at Rf 0.50 with chromatograms of folic acid, and also with chromatograms of aminopterin and leucovorin due to the presence of folic acid in these products. This component was not found in *a*-methopterin strips. With leucovorin strips additional growth spots occurred at Rf 0.63 and Rf 0.83. The latter corresponds to values reported for citrovorum factor but the former is at present unidentified.

Inhibition was demonstrated in medium containing folic acid by chromatograms of *a*-methopterin at zones corresponding to Rf values reported by Nichol and associates[8] for 4-aminopteroylglutamic acid, 4-amino, 10-methylpteroic acid, 4-amino, 10-methylpteroylglutamic acid, and 10-methylpteroylglutamic acid. Aminopterin gave a single zone of inhibition at Rf 0.25 (Fig. 3).

No areas of inhibition could be found in chromatograms of urine from 8 sprue patients, even when the concentration of folic acid in the medium was reduced to 1 Mμg per milliliter. Two treated patients excreted pteroic acid, folic acid, and presumably folic acid derivatives with Rf values of 0.60 or more.

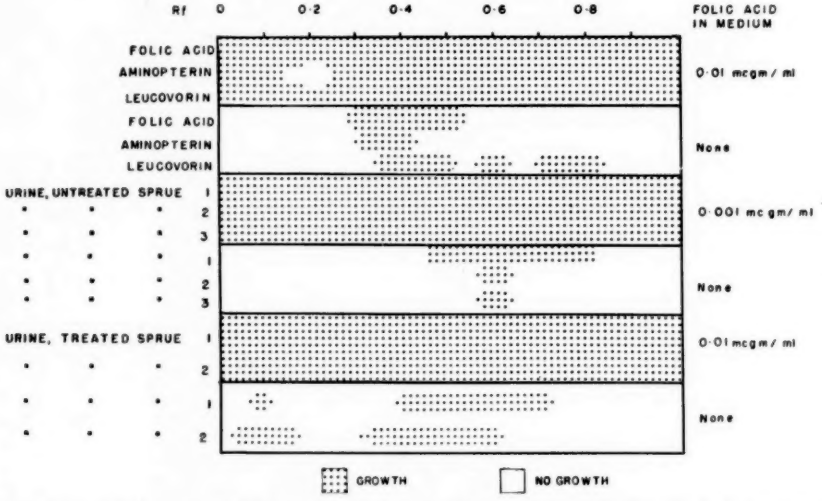

Fig. 4.—Diagram illustrating results obtained with chromatography of reference compounds and urine samples from 5 patients with sprue. An additional 3 untreated subjects gave similar results. For details see text.

All 8 patients with sprue excreted a growth promoting factor at the zone between Rf 0.55 and 0.65, corresponding to the unknown component of leucovorin with Rf 0.63. In 4 of the 6 patients in relapse this was the only spot found. Another untreated subject excreted growth promoting factors covering the zone from Rf 0.46 to 0.81, probably representing a small amount of folic acid as well as citrovorum factor and intermediate compounds. The sixth untreated subject excreted a growth factor with an Rf value of 0.67 and another at 0.84.

DISCUSSION

It is often difficult to make a clear distinction between faulty intestinal absorption and rapid removal of a test substance from the blood due to tissue depletion and other factors. This is particularly true in the case of sprue and folic acid because attempts to saturate the tissues by parenteral dosage prior

to testing causes clinical improvement. We have noted clinical improvement and reticulocytosis up to 10 per cent following a single 5 mg. dose of folic acid. In the present study the 5-hour urinary folic acid excretion value after a single oral dose was compared with the excretion after I.V. administration 2 to 6 days later. The data following intravenous dosage suggest some tissue depletion since untreated subjects retain about 12 per cent more folic acid than normals. If faulty intestinal absorption were the only abnormality in these patients similar results would be expected in the two groups of subjects.

In the light of this knowledge the wide disparity between the urine values for normal and untreated subjects after oral dosage would indicate malabsorption. Tissue depletion is apparently not sufficient to account for the twenty-five-fold reduction in excretion found in the untreated sprue patients.

It is perhaps significant to note that patients with tropical sprue in remission show normal excretion values both with oral and intravenous administration. This is taken as evidence that these patients do not have a permanent specific defect in the absorption of folic acid at the dosage used, nor do they have tissue depletion. However, the possibility remains that malabsorption for folic acid could exist at lower concentrations since the 5 mg. dose greatly exceeds the estimated daily requirement of less than 1 mg.[11] It has been reported that infants with megaloblastic anemia will respond to folic acid in doses as low as 200 and 500 μg per day.[12] It has been shown that pteroylhexaglutamylglutamic acid is effective by oral administration in treating tropical sprue[13] so that impairment of conjugase activity is probably not a feature in the faulty absorption of naturally occurring folic acid compounds. However, as Girdwood[3] has stated, it seems unlikely that sprue patients could absorb folic acid conjugates normally, while absorbing the free folic acid poorly. Although the data do not bear directly on the question, evidence that sprue patients have increased requirements for folic acid stems from the observations that they frequently relapse without a dietary supplement.

Serum determinations of folic acid activity after oral dosage likewise reveal a significant difference between the peak values of normal and untreated patients compatible with malabsorption. Values for treated subjects were virtually identical with the normals. Dilution of the test material by the relative increase in plasma volume of the anemic subject was considered but this is not an adequate explanation for the wide differences observed. Calculations indicate that the plasma volume of an average anemic sprue patient (weight 100 pounds) is not remarkably different from that of an average nonanemic control (weight 175 pounds).

Although the number of observations is small the serum concentration values after intravenous administration (Fig. 2) lend support to the concept that rapid clearance is not a factor in producing the flat uptake curve seen in the serum after oral dosage.

Evidence that impaired absorption is the main disturbance may also be seen in the tests of folic acid excretion before and during treatment. There was only a slight increase in excretion following intravenous dosage after

therapy, whereas the urinary excretion following oral dosage was approximately 30 times greater than before.

The assay of leukocyte folic acid affords a method of direct observation on cellular stores and serves as a check on the previous findings with oral and intravenous administration. The data indicate a statistically significant decrease in the mean folic acid content of leukocytes in patients with tropical sprue in relapse. However, the fact that the leukocyte folic acid concentration of some patients lies within the lower limit of the normal range tends to support the indirect observations made earlier that tissue depletion is not great. As noted above, patients in relapse retained only 12 per cent more of the intravenous dose than did normal subjects.

Observations were also made on leukocytes from 3 persons with chronic granulocytic leukemia, giving high values, as previously reported by Swendseid and associates. Our highest result was 175.7 Mμg per milliliter of leukocytes; this was considerably above our normal range but not as high as others have found. It may be of interest that Beard and associates[14] have found abnormally high levels of serum B_{12} in such patients. The white cell folic acid concentration in a man suffering from pernicious anemia in relapse was less than 24.2 Mμg per milliliter, this being the minimum reading possible with the standard curve. A serum sample drawn at the same time showed a vitamin B_{12} concentration of zero. These findings tend to support the concept of Tasker[15] and others that there may be a correlation between body stores of folic acid and B_{12}.

Several considerations led to a search for possible competitive inhibitors of folic acid. For example, there is some resemblance between tropical sprue and the clinical picture produced by certain folic acid analogs. Thus aminopterin may produce aphthous ulcers in the buccal mucosa, glossitis, diarrhea, leukopenia, and macrocytic anemia.[16] Hamilton and associates[17] have reported that dogs treated with a folic acid antagonist (''DDMP'') develop diarrhea, ulcerative lesions in the small intestine, skin pigmentation, and megaloblastic changes in the marrow. Similar results have been noted in human beings.[18] Interest has also been centered on the possible role of bacterial products synthesized in the blind loops, and diverticula of patients with the so-called ''pouch P.A.,'' who respond to antibiotic therapy. Several authors have reported an inhibitor in the plasma of patients with pernicious anemia which interferes with the maturation of megaloblasts in marrow cultures.

The present study failed to demonstrate any substance in the urine of sprue patients inhibitory to the growth of *S. faecalis.* The possibility remains that substances exist which may inhibit growth of human cells and not those of *S. faecalis.*

SUMMARY

1. Evidence is presented that there is marked malabsorption of folic acid in patients having tropical sprue in relapse, and that the tissue levels are moderately reduced.

2. The question remains unanswered as to whether malabsorption is the cause or the effect of folic acid deficiency. However, since folic acid absorp-

tion improves when the absorption of other substances improves it appears likely that this malabsorption may be only a part of intestinal dysfunction.

3. Measurement of serum levels after oral and intravenous dosage and measurement of leukocyte folic acid revealed no appreciable differences between normal persons and patients with sprue in remission. There does not appear to be a permanent specific defect in the absorption of folic acid by patients with sprue comparable to the malabsorption of vitamin B_{12} by patients with pernicious anemia.

4. No evidence has been found that sprue patients in remission require increased amounts of folic acid, other than the well-known clinical observations that they tend to relapse without dietary supplements.

5. A survey of urines from 8 patients failed to reveal substances inhibitory to the growth of the folic acid-dependent organism *S. faecalis.*

The technical assistance of Sergeant First Class Wallace G. Fee and Master Sergeant Kenneth S. Moran is gratefully acknowledged.

REFERENCES

1. Darby, W. J., and Jones, E.: Treatment of Sprue With Synthetic L. casei Factor ("Folic Acid," Vitamin "M"), Proc. Soc. Exper. Biol. & Med. **60:** 259-260, 1945.
2. Spies, T. D., Garcia-Lopez, G., Menendez, J. A., Minnich, V., and Koch, M. B.: The Effect of Folic Acid on Sprue, South. M. J. **39:** 30-32, 1946.
3. Girdwood, R. H.: A Folic Acid Excretion Test in the Investigation of Malabsorption, Lancet **2:** 53-60, 1953.
4. Garcia-Lopez, G., Spies, T. D., Menendez, J. A., and Lopez-Toca, R.: Folic Acid in the Rehabilitation of Persons With Sprue, J. A. M. A. **132:** 906-911, 1946.
5. Gardner, F. H.: A Malabsorption Syndrome in Military Personnel in Puerto Rico, A. M. A. Arch. Int. Med. **98:** 44-60, 1956.
6. Denko, C. W.: Pteroylglutamic Acid Clearance in Normal Adults, J. Appl. Physiol. **3:** 559-562, 1951.
7. Swendseid, M. E., Bethell, F. H., and Bird, O. D.: The Concentration of Folic Acid in Leukocytes. Observations on Normal Subjects and Persons With Leukemia, Cancer Res. **11:** 864-867, 1951.
8. Nichol, C. A., Zakrzewski, S. F., and Welch, A. D.: Resistance to Folic Acid Analogues in a Strain of Streptococcus faecalis, Proc. Soc. Exper. Biol. & Med. **83:** 272-277, 1953.
9. Fountain, J. R., Hutchison, D. J., Waring, G. B., and Burchenal, J. H.: Persistence of A-methopterin in Normal Mouse Tissues, Proc. Soc. Exper. Biol. & Med. **83:** 369-373, 1953.
10. Swendseid, M. E., Swanson, A. L., Meyers, M. C., and Bethell, F. H.: The Nutritional Status of Folic Acid in Persons With Leukemia and Its Possible Relation to Effects of Aminopterin Therapy, Blood **7:** 307-310, 1952.
11. Blanck, F. C. (editor): Handbook of Food and Agriculture, New York, 1955, Reinhold Publishing Corporation.
12. Woodruff, C. W., Peterson, J. C., and Darby, W. J.: Citrovorum Factor and Folic Acid in the Treatment of Megaloblastic Anemia of Infancy, Proc. Soc. Exper. Biol. & Med. **77:** 16-18, 1951.
13. Suarez, R. M., Welch, A. D., Heinle, R. W., Suarez, R. M., Jr., and Nelson, E. M.: Effectiveness of Conjugated Forms of Folic Acid in the Treatment of Tropical Sprue, J. LAB. & CLIN. MED. **31:** 1294-1304, 1946.
14. Beard, M. F., Pitney, W. R., and Sanneman, E. H.: Serum Concentration of Vitamin B_{12} in Patients Suffering From Leukemia, Blood **9:** 789-794, 1954.
15. Tasker, P. W. G.: Correlation of Serum-Vitamin B_{12} Levels and Urinary Folic Acid in Nutritional Megaloblastic Anemia, Lancet **2:** 61-63, 1955.
16. Farber, S.: Some Observations on the Effect of Folic Acid Antagonists on Acute Leukemia and Other Forms of Incurable Cancer, Blood **4:** 160-167, 1949.
17. Hamilton, L., Philips, F. S., Sternberg, S. S., Clarke, D. A., and Hitchings, G. H.: Hematological Effects of Certain 2, 4-Diaminopyrimidines Antagonists of Folic Acid Metabolism, Blood **9:** 1062-1081, 1954.
18. Murphy, M. L., Ellison, R. R., Karnofsky, D. A., and Burchenal, J. H.: Clinical Effects of the Dichloro and Monochlorophenyl Analogues of Diaminopyrimidine Antagonists of Folic Acid, J. Clin. Invest. **33:** 1388-1396, 1954.

GLYCOPROTEINS OF NORMAL HUMAN SERUM AS SEPARATED BY ELECTROPHORESIS ON STARCH

OTTO W. NEUHAUS, PH.D., AND MARCIA LETZRING, B.S.
DETROIT, MICH.

THE pattern of serum glycoprotein following electrophoresis is being increasingly studied as a means of observing serum protein changes in disease.[1-9] At the present time the glycoproteins are visualized either by staining the protein-bound polysaccharides on paper electropherograms with the periodic acid-Schiff (PAS) technique[10] or by determining the content of hexosamines in the separated zones.[2, 3] Such patterns have been evaluated densitometrically and by elution. A lack of specificity[1] and a high background coloration[4] are characteristic difficulties in the paper electrophoresis—PAS method. When glycoproteins are evaluated by their content of hexosamines, determinations are made on the separated zones of paper electropherograms.[2, 3] The paper segments are hydrolyzed and the hexosamine content measured by a modified Elson and Morgan procedure. The most striking characteristic of such data is the absence of a significant amount of hexosamine in the albumin peak; otherwise the data are similar to those obtained by the PAS procedure.

The electrophoretic separation of serum on a starch medium offers the unique opportunity for comparing a glycoprotein curve, measured as hexosamines, with the total protein pattern from the same electrophoretic run. In this paper results are presented for the separation on starch of individual and pooled human sera in an effort to help establish a normal pattern of serum glycoproteins.

METHOD

With the exception of one sample of pooled normal human serum, which had been stored in the frozen state for 6 months, all serum samples were used on the same day as drawn to obviate any changes that might occur either because of storage or the freezing and thawing process.

Zone Electrophoresis.—The technique used here is that of Kunkel.[11] Potato starch,* washed twice with a barbital buffer of pH 8.6 and 0.1 ionic strength, was made into a thick suspension with the buffer and then poured into a plastic mold 15 × 3 × ⅝ inches. After excess buffer was removed with filter paper, the serum sample (3 ml.), previously made into a paste with starch, was packed into a slot approximately 5 inches from the cathodic end of the starch block. Cellulose sponges were used to connect the ends of the block with the electrode vessels. After an initial equilibration period of one hour, 250 v.

From the Department of Anatomy and Physiological Chemistry, Wayne State University College of Medicine, Detroit, Mich.

This project was aided by grants from the United States Public Health Service (A-523 [C8]) and the Michigan Chapter Arthritis and Rheumatism Foundation.

Received for publication April 1, 1957.

*Amend Drug and Chemical Co., Inc., New York, N. Y., or Fisher Scientific Co., Pittsburgh, Pa.

were applied which furnished a current of 50 to 70 Ma. The electrophoretic separation as well as the initial equilibration period were conducted at 5° C. After 24 hours the starch was cut into 1 cm. segments each of which was then eluted with 5 ml. of a 1 per cent solution of salt.

Determination of Protein and Hexosamine.—Aliquots of the eluates were analyzed for protein by the method of Lowry.[12] For the determination of hexosamine, 1 ml. aliquots were first dried, hydrolyzed for 4 hours with 3 N HCl and dried in vacuo over NaOH. Then the isoamyl alcohol extraction procedure described in detail elsewhere*[13] was used on the neutralized hydrolysates.

For total serum hexosamines 1.0 ml. aliquots of a 1:100 dilution of serum were dried, hydrolyzed for 4 hours in 3 N HCl, and treated as described for the determination of hexosamine.

RESULTS

Interference in the recovery of hexosamine by partial hydrolysates of bovine serum albumin using the isoamyl alcohol extraction procedure has been previously reported.[13] To test the effectiveness of this procedure with serum, the recovery of 10 gammas of added glucosamine from 0.01 ml. of human sera was also studied. Ninety-five per cent of added glucosamine was recovered. The absorption curves of the chromogen for glucosamine, serum, and serum with added glucosamine were identical, indicating the absence of interference from nonglucosamine reactive substances of serum.

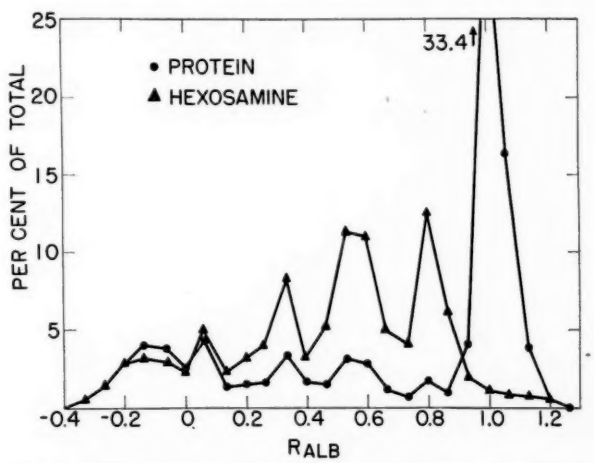

Fig. 1.—Glycoprotein pattern obtained by the electrophoretic separation of human serum.

The average total hexosamines was found to be 88 mg. per cent, a value which is in agreement with that of others.[2, 3, 8] A typical electrophoretic pattern obtained with pooled normal human sera is presented in Fig. 1. The protein and hexosamine contents of each segment of starch are plotted as per cent of the total. Each centimeter of starch is expressed as a fraction, $R_{albumin}$, which is defined as the distance migrated divided by the distance of the albumin peak from the origin. Points anodic to the origin are given positive values; cathodic ones are negative.

*This technique does not distinguish galactosamine from glucosamine.

The areas bounded by the conventional zones of the protein and glycoprotein curves were measured with a planimeter. The relative proportions of each zone are presented in Table I. No values are given for hexosamine in the albumin fraction as the amount found is insignificant (Fig. 1).

TABLE I. TOTAL PROTEIN AND GLYCOPROTEIN PATTERN OF NORMAL HUMAN SERUM

SERUM	TOTAL SERUM		PROTEIN (PER CENT)					HAm† (PER CENT)			
	PROTEIN* (GM./100 ML.)	HAm† (MG./100 ML.)	ALB.	α_1	α_2	β	γ	α_1	α_2	β	γ
Frozen pooled	7.4	94.5	56.6	2.6	9.5	8.5	22.7	25.4	35.7	15.8	23.0
Fresh pooled	7.1	81.3	54.2	5.3	10.2	11.5	18.8	27.2	32.2	22.5	18.1
ON	7.0	81.3	58.5		9.4	10.4	21.7	22.0	31.8	21.0	25.2
GL	7.6	96.3	55.2	5.1	11.1	11.8	16.8	32.1	33.6	18.9	15.4
ML	7.4	73.8	58.0	3.8	10.6	10.4	17.2	24.1	38.6	21.0	16.3
RF	7.9	100.0	52.5	4.0	7.5	10.1	25.9	27.1	29.7	19.9	23.3
AO	8.1	88.7	51.1	5.5	10.7	14.1	18.6	30.0	29.7	23.2	17.1
Average‡	7.5	88.0	54.2	4.7	9.9	11.4	19.8	27.1	32.6	21.1	19.2
s.d.	±0.4	±9.5	±2.6	±1.3	±1.3	±1.5	±3.4	±3.7	±3.1	±1.6	±4.0

*Total proteins were determined by Dr. B. Zak, Department of Pathology, Wayne State University College of Medicine, using a biuret method. [11]

†HAm is used to indicate hexosamine.

‡Data of frozen pooled sera not included.

TABLE II. GLYCOPROTEIN PATTERNS FROM PAPER ELECTROPHORESIS

METHOD	GLYCOPROTEINS (PER CENT)					REFERENCE
	ALB.	α_1	α_2	β	γ	
PAS* densitometric	13.1	17.9	27.9	23.8	17.2	Björnesjö[1]
PAS* densitometric	9.85	15.06	27.27	33.30	14.52	Magalini[6]
PAS* densitometric	25.0	15.0	19.0	21.0	20.0	Grassi[7]
PAS* densitometric	9.8	14.6	29.3	26.8	19.5	Shetlar†[15]
PAS* elution	8.7	21.2	36.0	25.2	8.2	Laurell[4]
Hexose	10.9	13.4	29.4	26.0	20.2	Shetlar†[15]
HAm‡	--	29.2	23.9	25.8	21.1	Goa†[3]
HAm‡	--	20.6	29.5	20.5	29.2	Bollet†[2]

*Stained with periodic acid-Schiff reagent.

†The data of these authors were originally given in mg. per 100 ml. of serum and have been recalculated as per cent of total.

‡HAm represents hexosamines.

DISCUSSION

Available data on the electrophoretic distribution of serum glycoproteins,[1, 4, 6, 7, 15] measured by the PAS stain, exhibit some discrepancies in the albumin and gamma zones (Table II). Some variation is undoubtedly the result of interference by noncarbohydrate substances and individual differences in staining technique. Despite such variabilities the data obtained are in general agreement with glycoprotein patterns based on hexosamine and hexose determinations.[1, 15] With the availability of a simplified analysis for hexosamines the disadvantages of PAS staining can be obviated. The use of hexosamine as a criterion is especially pertinent because of the increasing efforts to follow changes in total serum hexosamine in disease.[8, 9] The data of Bollet[2] and Goa[3] (Table II) combined with those of this paper are a start in developing a clearer picture of the normal distribution of serum glycoproteins as reflected in the distribution of hexosamines.

SUMMARY

Normal human sera were separated electrophoretically using starch as the stabilizing medium. Determinations for protein and hexosamine were made on the consecutive eluates. The average relative distribution of hexosamines in the electrophoretically separated zones was 27.1 per cent in alpha-1, 32.6 per cent in alpha-2, 21.1 per cent in beta, and 19.2 per cent of the total hexosamine in the gamma fraction.

The interest and advice of Drs. A. J. Bollet and B. Zak are gratefully acknowledged.

REFERENCES

1. Björnesjö, K. B.: Staining of Protein-Bound Serum Polysaccharide in Electrophoresis Strips, Scandinav. J. Clin. & Lab. Invest. **7**: 153, 1955.
2. Bollet, A. J.: The Hexosamine Content of the Serum Globulins in Normal and Pathological Sera, J. Clin. Invest. **36**: 51, 1957.
3. Goa, J.: On Protein-Bound Carbohydrates in Human Serum, Scandinav. J. Clin. & Lab. Invest. **7** (Suppl.): 22, 1955.
4. Laurell, C. B., and Skoog, N.: Quantitative Determination of Glucoprotein Pattern of Normal Serum After Electrophoretic Separation on Filter Paper, Scandinav. J. Clin. & Lab. Invest. **8**: 21, 1956.
5. Laurell, H.: A Paper Electrophoretic Study of the Effect of ACTH and Cortisone on the Protein-Bound Serum Polysaccharides in Some Patients With Rheumatoid Arthritis, Ulcerative Colitis, etc., Ciba Foundation Symposium, Boston, 1956, Little, Brown, & Co., pp. 58-76.
6. Magalini, S. I., Stefanini, M., Nogueira de Magalhaes, M., Angelopoulos, B., and Kistner, S.: Electrophoretic Distribution of Serum Glycoproteins in Some Hemorrhagic States, J. LAB. & CLIN. MED. **47**: 923, 1956.
7. Grassi, B., Andres, G., and Andreoli, M.: Protidogramma e Glicoprotidogramma Serico e Liquorale in Soggetti Nomali ed in Meningonevrassitici, Rass. fisiopat. clin. **27**: 519, 1955.
8. Winzler, R. J.: Determination of Serum Glycoproteins, in: Methods of Biochemical Analysis, edited D. Glick, vol. 2, New York, 1955, Interscience Publishers, Inc., pp. 279-311.
9. Greenspan, E.: Clinical Significance of Serum Mucoproteins, Advances Int. Med. **7**: 101, 1955.
10. Köiw, E., and Grönwall, A.: Staining of Protein-Bound Carbohydrates After Electrophoresis of Serum on Filter Paper, Scandinav. J. Clin. & Lab. Invest. **4**: 244, 1952.
11. Kuńkel, H. G.: Zone Electrophoresis, in: Methods of Biochemical Analysis, edited D. Glick, vol. 1, New York, 1954, Interscience Publishers, Inc., pp. 141-170.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.: Protein Measurement with the Folin Phenol Reagent, J. Biol. Chem. **193**: 265, 1951.
13. Neuhaus, O. W., and Letzring, M.: A Determination of Hexosamines in Conjunction With Electrophoresis on Starch, Anal. Chem. **29**: 1230, 1957.
14. Wolfson, W. Q., Cohn, C., Calvary, E., and Ichiba, F.: Studies in Serum Proteins V. A Rapid Procedure for the Estimation of Total Protein, True Albumin, Total Globulin, Alpha Globulin, Beta Globulin and Gamma Globulin in 1.0 ml. of Serum, Am. J. Clin. Path. **18**: 723-730, 1948.
15. Shetlar, M. R., Cahill, C., Stidworthy, G., and Shetlar, C. L.: Comparison of Continuous and Strip Paper Electrophoresis Technics for Study of Serum Glycoproteins, Proc. Soc. Exper. Biol. & Med. **93**: 44, 1956.

ACID MUCOPOLYSACCHARIDE EXCRETION IN THE URINE OF CHILDREN

CLAYTON RICH, M.D., NICOLA DIFERRANTE, M.D.,* AND REGINALD M. ARCHIBALD, M.D., PH.D.
NEW YORK, N. Y.

URINARY acid mucopolysaccharide (APS) excretion has been measured in normal adults[1, 2] and children[1] and in patients with lupus erythematosus,[3] rheumatoid arthritis,[4] and diabetes.[5] The APS of normal human urine has been shown to be mainly chondroitin sulfate.[6] It was observed that normal children excreted amounts of APS which were several times higher than values observed in normal adults.[1] This report describes a study of its excretion in the urine of normal children, and of children with retarded or accelerated growth.

MATERIALS AND METHODS

Except for a few hospitalized children, the subjects studied were outpatients, followed in the growth and development clinic conducted by one of us (R. M. A.), and normal siblings of a large number of these patients. Each patient had a complete history and physical examination and an x-ray of the wrists for interpretation of bone age.[7] One or more 24-hour urine sample from each patient was analyzed for APS (which is expressed as milligrams of glucuronic acid per 24 hours)[1] and creatinine.[8] The patient or his parents were carefully instructed before, and questioned after, the collection of urine and only those samples which were considered to be complete and to have been continuously refrigerated were accepted. Paper chromatography was carried out in the propanol, phosphate buffer system described by Kerby.[2]

Analyses of the Data.—Notwithstanding a history of complete collection, each sample was examined by an independent means to assess the reliability of the collections. This was done by dividing the patients into 3 groups: those in whom determinations from 2 different urine samples were in close agreement, those in whom the creatinine excretion of a single urine collection was well within the normal range, and those in whom the completeness of the urine collection could not be verified. The first 2 groups were considered reliable.

The entire group studied consisted of 61 male and 26 female children from 3 to 16 years of age. In 49 of these patients, analyses were carried out for APS or creatinine on 2 or more 24-hour urine specimens collected within a period of 6 months. From 39 patients the values were within 15 per cent of their average, and these were accepted as representing complete collections. These patients were designated Group I.

Of Group I, 24 subjects were considered to be normal in that there was no diagnosis of a metabolic or developmental abnormality, and in that the bone age differed by less than 1.5 years from the chronological age. Eighteen of the normal subjects were male and 6 were female. Of the patients in Group I, only the normal males were numerous enough to permit separate statistical analysis. The normal daily creatinine excretion is shown for this subgroup in Fig. 1.

From The Rockefeller Institute for Medical Research, New York, N. Y.
Received for publication June 10, 1957.
*Present address: Department of Biochemistry, University of Rochester, Rochester, N. Y.

The remaining 38 subjects in this study had only one urine specimen analyzed for APS and creatinine. Of the 23 normal males in this group, 17 had values for creatinine excretion which were within one standard error of the normal as determined by the line of regression for the data in Fig. 1. These 17 subjects were designated Group II. The normal males in Groups I and II were analyzed separately in regard to APS and creatinine excretion related to age, body weight, and surface area. They were found to be homogeneous in these respects and were therefore considered together for all further analyses.

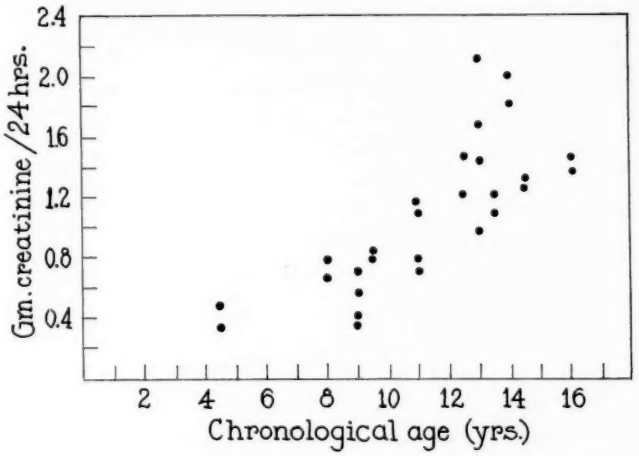

Fig. 1.—The creatinine excretion of normal male children.

In 31 patients, the accuracy of urine collection could not be verified. These subjects were designated Group III. All of the urine collections from patients in Group III were considered to be incomplete, but to represent a major fraction of the 24-hour period.

Whenever inspection indicated a linear correlation between a measurement and the subject's age, a line of regression was determined by the method of least squares. Standard statistical analysis was employed.[9]

TABLE I. THE CREATININE COEFFICIENTS OF NORMAL MALE CHILDREN

AGE	CREATININE COEFFICIENT (MG./24 HR./KG.) MEAN ± S.D.
<7	14.5 and 19.1
7- 8	23.4 ± 4.6
9-10	22.0 ± 2.7
11-12	24.2 ± 8.7
13-14	24.9 ± 5.0
15-16	28.0 ± 4.4

RESULTS

The creatinine coefficient of the normal males was found to vary widely in any age group. The mean values at different ages are indicated in Table I. It can be seen that the creatinine coefficients of the youngest patients were possibly lower than the normal adult values of 20 to 26. This is consistent with numerous reports of low creatinine coefficients in early childhood.[10] However, in the children from 7 to 16 years of age, there was no statistically significant change in the creatinine coefficient with age. The few values obtained for females indicated lower creatinine coefficients for this sex at all ages (16.3 ± 2.6 mg./24 hr./Kg.).

The 24-hour excretion of APS in normal males and females (Groups I and II) is indicated in Fig. 2. In males there was a progressive increase in APS excretion. This was maximum between the thirteenth and fifteenth year. The highest values of 18 to 23 mg. were 3 times the normal adult value[1, 2] and as high or higher than the values seen in adults with pathologic states associated with increased APS excretion.[3, 4] The values recorded for females were lower than those of males but showed a similar increase during adolescence. This increase occurred, as expected, at an earlier age in female than in male children.

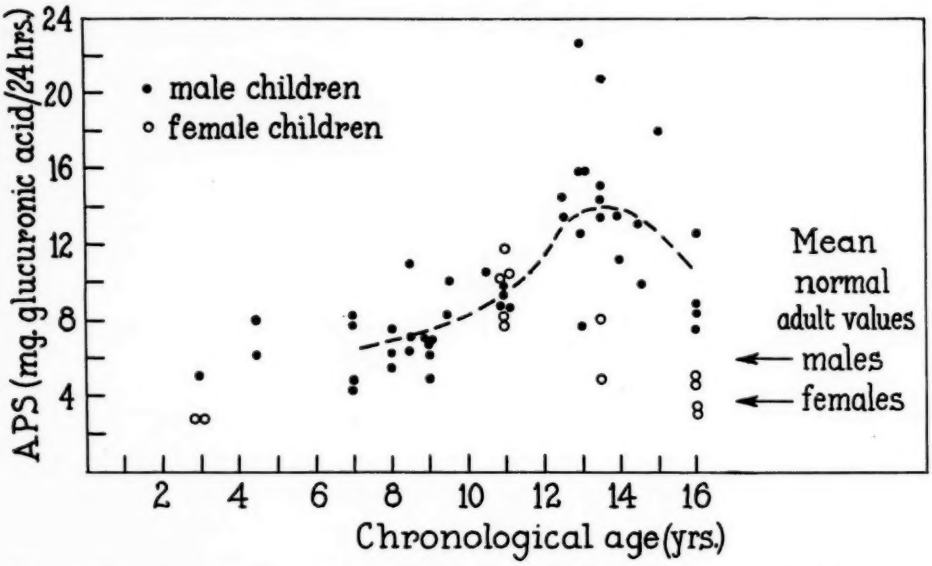

Fig. 2.—The acid mucopolysaccharide excretion of normal children. (The dashed line joins the mean of each 2-year period for male children.)

TABLE II. THE LINEAR CORRELATIONS BETWEEN THE RATIO OF APS EXCRETION TO WEIGHT OR CREATININE EXCRETION AND AGE

GROUP ANALYZED	ABCISSA (X-AXIS)	ORDINATE (Y-AXIS)	LINE OF REGRESSION ± S.E.	COEFFICIENT OF CORRELATION ± S.D.
Normal males Groups I and II	Chronological age (yr.)	$\frac{\text{APS (mg./24 hr.)}}{\text{wt. (Kg.)}}$	$y = 1.69 - 0.047 \times \pm 0.33$	$- 0.32 \pm 0.12$
Same	Same	$\frac{\text{APS (mg./24 hr.)}}{\text{creatinine (Gm./24 hr.)}}$	$y = 18.8 - 0.73\,x \pm 2.3$	$- 0.64 \pm 0.09$
All males (Line for Fig. 3)	Bone age (yr.)	Same	$y = 17.9 - 0.71\,x \pm 2.3$	$- 0.70 \pm 0.07$
All females (Line for Fig. 4)	Same	Same	$y = 16.7 - 0.76\,x \pm 1.8$	$- 0.90 \pm 0.04$

It is useful to relate any determination from children of different ages to some index to compensate for their continuously increasing body mass. The commonly used indices are weight, height, surface area, and creatinine excretion. The ratio of APS excretion to height or surface area correlated

poorly with age. When the ratio of APS excretion to weight or creatinine excretion was related to age in this group of normal males, the linear correlations presented in Table II were obtained. The calculations from this table

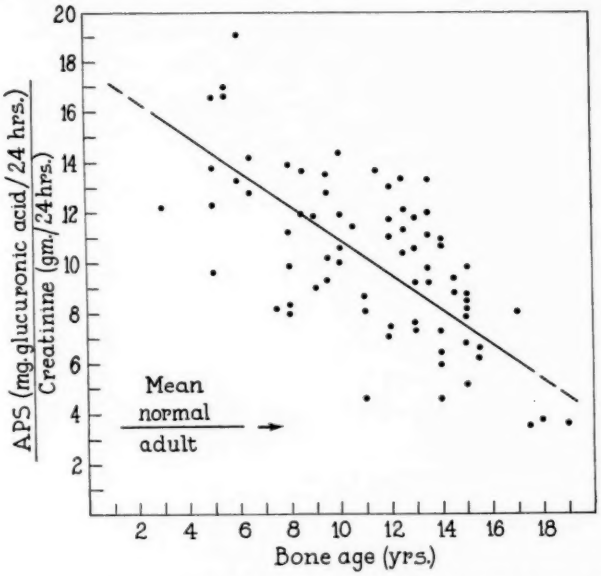

Fig. 3.—The acid mucopolysaccharide-creatinine ratio related to bone age of all male children studied. (Data from hypothyroid children are not included.)

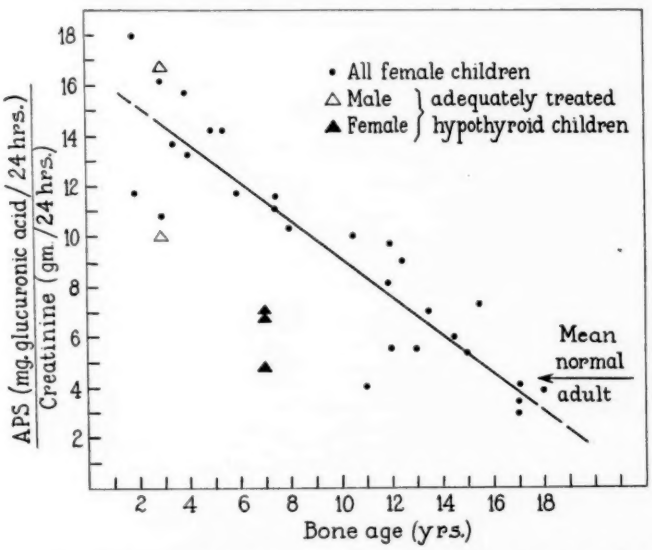
• All female children
△ Male } adequately treated
▲ Female } hypothyroid children
Mean normal adult
APS (mg. glucuronic acid/24 hrs.) / Creatinine (gm./24 hrs.)
Bone age (yrs.)

Fig. 4.—The acid mucopolysaccharide-creatinine ratio related to bone age of all female children studied. (Data from hypothyroid children, though plotted, are not included in the statistical analyses.)

show that the correlation of the APS:weight ratio with age was of little significance, but that the correlation of the APS:creatinine ratio with age was significant. As the age increased, there was a regular decrease of the amount

of APS excreted per unit of creatinine excreted. This correlation was improved when bone age was used in place of chronological age. The use of creatinine excretion as an index permitted the inclusion in the analysis of patients in Group III.

In Figs. 3 and 4 the APS:creatinine ratio was compared with the bone age for all the males and females, respectively, in the study. There was an obvious difference between the lines of regression drawn from the data from these 2 groups. In both groups the APS:creatinine ratio decreased with age at approximately the same rate, but in females this ratio was lower than in males at all ages. The patients of each sex with advanced or retarded bone age were analyzed separately, and found to show no significant difference from the normal subjects when bone age, but not when chronological age, was used. In every case, the correlation between the APS:creatinine ratio and the bone age was superior to the similar correlation with the chronological age.

The diagnoses other than advanced or delayed growth were enuresis (9 times), dwarfism with or without pituitary or gonadal insufficiency (5 times), alopecia totalis or areata (4 times), hypothyroidism under treatment (3 times), and each of the following once: anorexia nervosa, Osgood-Schlatter's disease, and adequately treated thyrotoxicosis. There was no difference between the APS:creatinine ratio of any of these patients and the others of their sex except in the case of the treated hypothyroid subjects, who had APS:creatinine ratios conspicuously below the values for their ages. (Except in one instance, these ratios deviated from the normal by more than 2 standard errors and none were included in the statistical analyses. Their positions are indicated in Fig. 4.)

The APS isolated from a number of urines which contained large amounts of APS and from several of the younger subjects would not pass by dialysis through a cellophane membrane. Its behavior during paper chromatography and subsequent reaction with toluidine blue was identical to that previously described for APS from normal urines.[1, 2, 6]

It was possible to extend these observations to a very early stage of life because of the interest and enthusiasm of Dr. Helen Ossofsky at the New York Hospital, who made available four 24-hour urine samples collected by catheter from each of 2 infants. A normal male one month old excreted 2.6 $\pm$ 0.5 mg. APS glucuronic acid and 0.044 $\pm$ 0.006 Gm. creatinine per day, and a 1.5-month old girl with osteogenesis imperfecta excreted 3.2 $\pm$ 0.3 mg. APS glucuronic acid and 0.032 $\pm$ 0.006 Gm. creatinine per day. The APS:creatinine ratios of these 2 infants were 59 and 97.

DISCUSSION

It has been shown that the urinary APS is not a product of the kidney or urinary tract[2] and further shown that in pooled urine from normal adults and children this APS is mainly chondroitin sulfate.[6] Chondroitin sulfate has also been demonstrated in normal human plasma.[11] The urinary excretion of APS is elevated in several diseases associated with abnormal metabolism of

mucopolysaccharide-rich connective tissues.[3, 4] It is reasonable to suppose that the urinary mucopolysaccharide excretion is a reflection of the metabolic state of the chondroitin sulfate containing tissues of the body.

The data presented in this study indicate that there is a regular decrease with age of the amount of APS excreted per unit of creatinine excreted. The ratio of APS:creatinine excretion attained adult values at about 17 years in females and 21 years in males. To the extent that these data reflect tissue acid mucopolysaccharide metabolism they indicate that an initially very active process gradually decreased as the growth rate fell with increasing age, to reach adult levels about the time when growth stopped.

SUMMARY

1. Acid mucopolysaccharide (APS) excretion has been described for children of both sexes whose ages were between 3 and 16 years. There was a gradual increase in APS excretion during childhood which became maximum during early adolescence.

2. The ratio of APS excreted per day to creatinine excreted per day was found to give a linear correlation with age. As age increased, a regular decrease occurred in the ratio of APS excretion to creatinine excretion.

3. There was no difference in the APS:creatinine ratio between normal children and children with advanced or delayed bone age if this ratio were correlated with bone age rather than chronological age.

REFERENCES

1. DiFerrante, N., and Rich, C.: The Determination of Acid Aminopolysaccharide in Urine, J. LAB. & CLIN. MED. **48:** 491-494, 1956.
2. Kerby, G. P.: The Excretion of Glucuronic Acid and of Acid Mucopolysaccharides in Normal Human Urine, J. Clin. Invest. **33:** 1168-1174, 1954.
3. DiFerrante, N., Robbins, W. C., and Rich, C.: Urinary Excretion of Acid Mucopolysaccharides by Patients With Lupus Erythematosus, J. LAB. & CLIN. MED. In Press.
4. DiFerrante, N.: Urinary Excretion of Acid Mucopolysaccharides by Patients With Rheumatoid Arthritis, Fed. Proc. **16:** 172-173, 1957.
5. Craddock, J. G. Jr., and Kerby, G. P.: Urinary Excretion of Acid Mucopolysaccharides by Diabetic Patients, J. LAB. & CLIN. MED. **46:** 193-198, 1955.
6. DiFerrante, N., and Rich, C.: The Mucopolysaccharide of Normal Human Urine, Clin. Chim. Acta. **1:** 519-524, 1957.
7. Flory, C. D.: Osseous Development in the Hand as an Index of Skeletal Development, Soc. for Research in Child Development Monographs. **1:** No. 3, 1936.
8. Peters, J. P., and Van Slyke, D. D.: Quantitative Methods in Clinical Chemistry, Baltimore, 1932, The Williams & Wilkins Company, Vol. II, p. 559.
9. Arkin, H., and Colton, R. R.: An Outline of Statistical Methods, ed. 3, New York, 1938, Barnes & Noble, Inc.
10. Hunter, A.: Creatine and Creatinine, London, 1928, Longmans Green & Co., Ltd.
11. Schiller, S., and Dewey, K. F.: Isolation of Chondroitinsulfuric Acid From Normal Human Plasma, Fed. Proc. **15:** 348, 1956.

THE EFFECT OF MORPHINE SULFATE UPON THE RENAL EXCRETION OF WATER AND SOLUTE IN MAN

SOLOMON PAPPER, M.D., LAWRENCE SAXON, M.D.,* MAURICE B. BURG, M.D., HAROLD W. SEIFER, M.D., AND JACK D. ROSENBAUM, M.D.
BOSTON, MASS.

THAT the administration of morphine may be followed by a decreased rate of urine formation has been known for a half century.[1] It is commonly stated that this change is mediated primarily by the release of antidiuretic hormone (ADH). Such statements derive largely from studies in which large doses of this alkaloid were administered to experimental animals.[2-9] Observations concerning the effects of therapeutic doses of morphine on the rate of urine flow, glomerular filtration rate, and solute excretion in normal man are so limited and contradictory[10-14] that a study of such effect was undertaken. The data presented demonstrate that morphine, when administered in usual therapeutic doses to healthy men, may decrease the rate of water diuresis without evident release of antidiuretic hormone. Such antidiuresis occurs concomitantly with a reduction in glomerular filtration rate and solute excretion.

METHODS

A water load was established on 22 occasions in 16 normal subjects by the oral administration of 20 ml. of tap water per kilogram of body weight. The water load was maintained throughout the experimental period by providing sufficient water after each voiding to restore body weight to the value attained after the initial loading. The subjects remained recumbent throughout the experiment except when they stood to void. After diuresis had begun, voided urine was collected at intervals of no greater than 30 minutes in all but a few experiments. After at least two periods of "maximal" diuresis, morphine sulfate was administered in a dose varying between 7.5 and 30 mg. subcutaneously or 5 to 10 mg. intravenously. Urine collections were continued for variable periods of time, generally for 105 to 250 minutes after drug administration.

Samples of venous blood were obtained during the height of diuresis and at the end of the study. Blood and urine were analyzed for their content of sodium, potassium, chloride, and creatinine by methods previously described from this laboratory.[15] Total osmolality was determined by freezing point measurement, employing a Fiske osmometer. Inulin and paraaminohippurate clearances were determined in 2 subjects.[16, 17] Equations given by Smith were employed for calculation of osmolal and free water clearances.[18]

RESULTS

1. The central nervous system effects of morphine were apparent within 30 minutes in all but 2 subjects. The manifestations varied in intensity and consisted of drowsiness, "weakness," relaxation, dizziness, and pupillary constriction. In Subject 13 very mild drowsiness and pupillary constriction were

From the Medical Service and Research Laboratory, Boston Veterans Administration Hospital, and the Departments of Medicine, Boston University School of Medicine and Tufts University School of Medicine, Boston, Mass.
Received for publication May 21, 1957.
*U. S. Public Health Service Research Fellow of the National Health Institute.

noted for the first time one hour after the morphine was given. Subject 16 had no subjective symptoms but his pupils became small at the end of an hour. Nine subjects developed nausea at some time during the experiment. Changes in blood pressure did not occur except in one subject who manifested postural hypotension on a single occasion.

2. In 17 of the 22 experiments, within 10 to 55 minutes after morphine administration, urine flow decreased by 2.1 to 6.6 ml. per minute (Table I). In 8 of these 17, the decrease in flow was observed within 30 minutes after morphine was given. The diminution in urine flow was *not* accompanied by a significant rise in urine osmolality. This decrease in diuresis was associated with a reduction in endogenous creatinine clearance varying between 10 and 41 ml. per minute in 16 of the 17 experiments.* In 16 of the 17 studies there was a decrease in total solute excretion varying between 83 and 476 μOsm per minute.

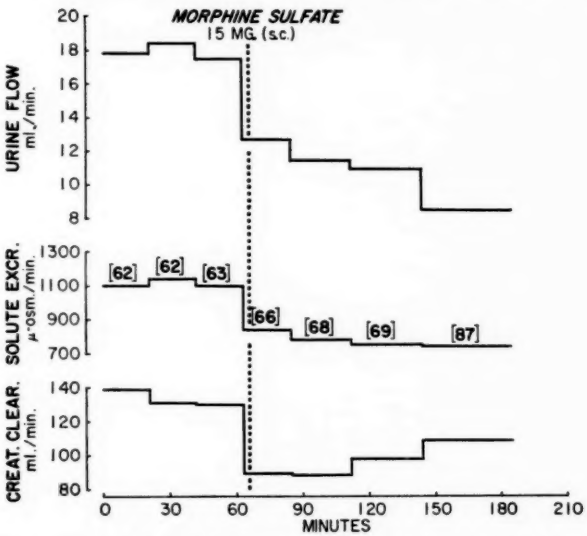

Fig. 1.—The effect of morphine sulfate (15 mg. subcutaneously) upon the renal excretion of water and solute in Subject 3. The numbers in brackets are the urine concentrations (mOsm./L.) for the respective periods.

There was little change in the urinary concentration of sodium and chloride when the rate of excretion of these ions decreased with the decline in flow, except in one experiment. The urinary concentration of potassium also remained fairly constant when flow diminished, but its rate of excretion decreased in all save 3 instances. As shown in Table I, there was a decrease in osmolal clearances in all but 2 experiments (12a and 12b) and free water clearance generally decreased concomitantly.

The concentration of total solutes and of sodium in serum, measured at the height of the initial diuresis and again at the end of the study, remained unchanged.

Data illustrating the entire course of 3 experiments are depicted in Figs. 1 to 3. In Subject 3 (Fig. 1), the initial prompt decrease in urine flow was associated with a striking reduction in creatinine clearance and a decline in

*In Subjects 4 and 8 inulin clearance as well as creatinine clearance was determined and comparable results obtained (Table I).

TABLE I. EXPERIMENTS IN WHICH MORPHINE SULFATE PRODUCED A DECREASE IN URINE FLOW OF MORE THAN 2 ML./MIN. WITHIN 10 TO 55 MINUTES OF DRUG ADMINISTRATION (GROUP I)

EXPERIMENT NO.	ELAPSED TIME (MIN.)	FLOW (ML./MIN.)	URINE								CLEARANCES		
			TOTAL SOLUTE		SODIUM		POTASSIUM		CHLORIDE				
			mOSM./L.	μOSM/MIN.	MEQ./L.	μEQ/MIN.	MEQ./L.	μEQ/MIN.	MEQ./L.	μEQ/MIN.	C_{Cr} (ML./MIN.)	C_{Osm} (ML./MIN.)	C_{H_2O} (ML./MIN.)
1a	40-60	15.7	58	910	4.9	77	10.2	160	9.0	141	140	3.3	12.4
7.5. mg.	60-80*	14.5	60	865	5.1	73	9.7	140	7.8	112	137	3.1	11.4
S.C.	80-105	12.7	58	736	3.8	48	8.8	112	5.5	69	137	2.7	10.0
1b	41-61	22.2	61	1,350	12.4	274	7.4	165	15.8	349	150	4.8	17.4
15 mg.	61-81*	20.4	61	1,240	11.8	241	7.4	151	14.8	301	144	4.5	15.9
S.C.	81-101	15.6	56	874	7.3	114	5.5	85	9.1	142	140	3.2	12.4
1c	40-60	20.9	50	1,040	8.4	176	5.7	120	10.3	214	141	3.8	17.1
15 mg.	60-80*	18.9	50	943	10.0	189	6.3	118	11.8	223	137	3.4	15.5
S.C.	80-100	15.2	54	821	9.6	145	5.9	90	9.6	146	128	3.0	12.2
2a	39-56	18.5	59	1,090	11.5	212	6.4	118	11.0	203	149	4.0	14.5
15 mg.	56-77*	17.6	61	1,070	11.9	209	7.0	123	11.1	196	143	3.9	13.7
S.C.	77-103	14.8	63	933	11.7	173	7.7	113	11.9	176	133	3.4	11.4
2b	45-65	18.5	52	962	7.8	145	4.8	84	7.0	130	149	3.4	15.1
15 mg.	65-76*	17.0	53	906	7.1	120	4.9	84	5.7	96	143	3.2	13.8
S.C.	76-98	14.6	56	827	7.0	104	6.5	97	6.3	92	124	2.9	11.7
2c	160-181	12.1	58	704	10.7	130	3.9	47	11.0	134	133	2.6	9.5
15 mg.	181-201*	11.8	63	740	10.9	128	3.4	40	10.9	128	127	2.7	9.1
S.C.	201-222	9.3	60	553	9.9	92	3.1	29	9.7	90	111	2.0	7.3
3	42-63	17.5	63	1,100	11.3	198	7.0	123	14.7	257	130	4.0	13.5
15 mg.	63-85*	12.7	66	840	13.5	172	7.3	93	18.4	235	89	3.0	9.7
S.C.	85-112	11.5	68	781	12.8	147	7.4	85	18.1	208	88	2.8	8.7
4	20-40	18.5	66	1,226	15.8	292	6.4	119	15.6	288	153†	4.7	13.8
15 mg.	40-60*	16.4	67	1,089	15.4	251	7.0	115	14.8	242	142	4.2	12.2
S.C.	60-80	16.4	63	1,029	14.6	240	7.1	116	13.7	224	151	4.0	12.4
5	40-60	19.7	49	965	5.9	116	6.9	136	6.2	123	143	3.5	16.2
15 mg.	60-81*	17.8	50	896	6.1	109	7.0	125	6.1	108	140	3.2	14.6
S.C.	81-101	14.3	50	719	4.9	70	5.8	84	4.7	67	132	2.6	11.7

6	40-60	14.6	79	1,150	13.4	196	13.8	201	15.8	231	128	4.2	10.4
30 mg.	60-81*	12.4	81	953	14.1	166	13.6	160	15.7	184	107	3.5	8.9
S.C.	81-102	9.0	76	648	10.4	89	11.4	97	12.4	106	91	2.3	6.7
7	40-60	18.4	50	920	4.1	75	8.5	156	7.0	129	150	3.3	15.1
30 mg.	60-80*	19.0	51	969	4.4	84	8.8	167	7.5	143	148	3.5	15.5
S.C.	80-100	16.1	52	837	4.2	67	9.2	148	7.0	112	138	3.0	13.1
8	40-60	15.1	73	1,094	9.6	144	8.5	128	11.6	175	167‡	4.0	11.1
30 mg.	60-80*	14.8	70	1,033	10.3	152	7.3	108	10.8	159	163	3.8	11.0
S.C.	80-100	12.8	73	921	9.0	113	7.3	92	9.6	121	157	3.4	9.4
9a	22-42	18.5	55	1,020	3.1	56	9.6	178	5.1	93	157	3.7	14.8
10 mg.	42-62*	14.8	53	772	2.6	38	4.9	73	4.2	62	139	2.8	12.0
I.V.	62-81	10.0	61	610	0.9	8	8.2	82	2.4	24	141	2.2	7.8
10	61-81	17.5	64	1,122	10.1	178	9.2	161	15.2	266	162	4.1	13.4
10 mg.	81-98*	14.3	69	995	10.1	146	10.7	152	16.9	241	140	3.7	10.6
I.V.	98-117	14.6	67	975	10.0	146	9.6	140	16.6	243	94	3.6	11.0
11	60-80	13.2	60	789	9.2	121	7.6	100	14.4	189	124	2.8	10.4
10 mg.	80-120*	8.5	61	525	5.7	49	7.4	63	10.5	90	105	1.9	6.6
I.V.	120-180	9.2	60	552	5.2	48	6.3	58	8.6	79	124	2.0	7.2
12a	41-61	19.8	58	1,150	8.4	165	8.0	159	9.1	179	192	4.2	15.6
7.5 mg.	61-71*	20.1	59	1,196	9.1	183	8.1	164	9.6	194	197	4.3	15.8
I.V.	71-96	17.0	62	1,052	9.4	159	8.1	138	10.1	172	182	3.8	13.2
12b	20-40	17.9	56	999	6.7	119	7.5	134	6.8	122	206	3.6	14.3
10 mg.	40-70*	16.9	58	986	7.8	133	7.8	124	7.6	131	195	3.5	13.4
I.V.	70-95	15.1	63	950	9.5	144	7.9	120	10.2	154	184	3.4	11.7

*Morphine administered at the beginning of this period except in Experiments 7, 8, and 9a, when it was given at the mid-point of the period.

†Corresponding values for inulin clearance were 128, 117, and 126 ml./min. Simultaneous PAH clearances were 681, 646, and 707 ml./min.

‡Corresponding values for inulin clearance were 158, 158, and 145 ml./min. Simultaneous PAH clearances were 592, 598, and 507 ml./min.

the rate of solute excretion. Approximately 2 hours after the administration of morphine, there was a further decline in urine flow with a rise in osmolality from 69 to 87 mOsm./L. This subject had nausea and vomited at the end of the experiment. Subject 7 (Fig. 2) had a prompt reduction in urine flow

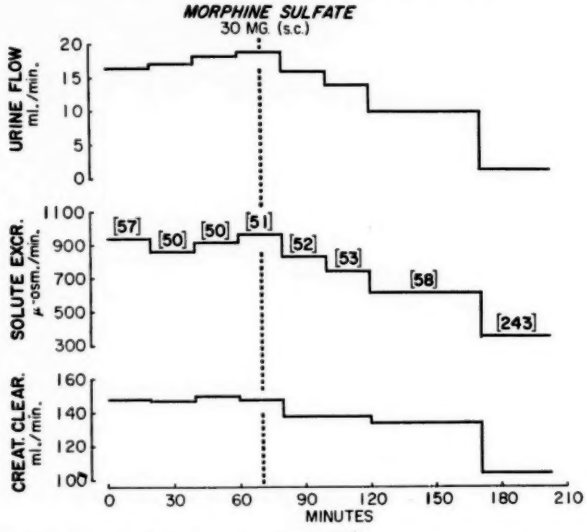

Fig. 2.—The effect of morphine sulfate (30 mg. subcutaneously) upon the renal excretion of water and solute in Subject 7. The numbers in brackets are the urine concentrations (mOsm./L.) for the respective periods.

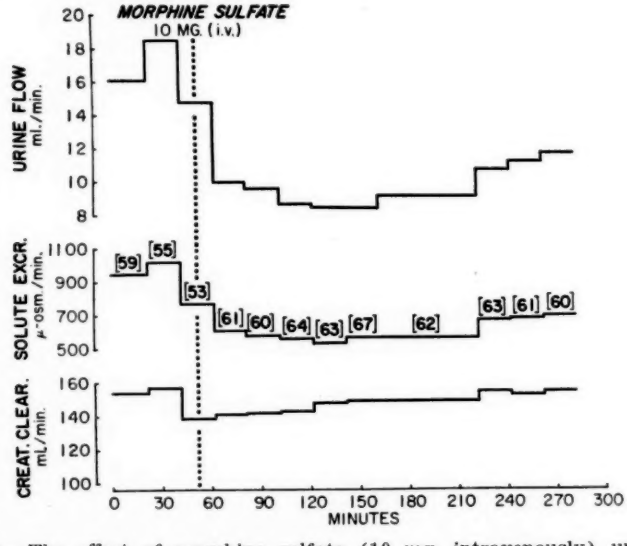

Fig. 3.—The effect of morphine sulfate (10 mg. intravenously) upon the renal excretion of water and solute in Subject 9a. The numbers in brackets are the urine concentrations (mOsm./L.) for the respective periods.

associated with a decline in creatinine clearance of 12 ml. per minute and a decreased rate of solute excretion but without a rise in urine osmolality. Approximately 130 minutes after morphine administration there was a striking

TABLE II. EXPERIMENTS IN WHICH MORPHINE SULFATE DID NOT PRODUCE A DECREASE IN URINE FLOW OF MORE THAN 2 ML./MIN. WITHIN 55 MINUTES OF DRUG ADMINISTRATION (GROUP II)

EXPERIMENT NO.	ELAPSED TIME (MIN.)	FLOW (ML./MIN.)	URINE								CLEARANCES		
			TOTAL SOLUTE		SODIUM		POTASSIUM		CHLORIDE				
			mOSM./L.	μOSM/MIN.	MEQ./L.	μEQ/MIN.	MEQ./L.	μEQ/MIN.	MEQ./L.	μEQ/MIN.	C_{Cr} (ML./MIN.)	C_{Osm} (ML./MIN.)	C_{H_2O} (ML./MIN.)
9b	42-64	13.9	53	735	3.7	51	10.1	140	5.8	81	149	2.7	11.2
5 mg.	64-84*	12.8	60	765	2.9	37	10.0	128	3.8	48	152	2.8	10.0
I.V.	84-104	11.9	59	699	2.4	29	9.5	112	3.3	39	149	2.5	9.4
13	20-40	16.4	69	1,134	12.9	211	8.2	135	14.2	233	159	4.1	12.3
10 mg.	40-60*	16.3	71	1,170	14.6	236	7.6	123	15.7	256	169	4.3	12.0
I.V.	60-80	14.7	75	1,095	16.9	249	6.9	101	18.1	269	165	4.0	10.7
14	49-77	11.5	62	715	5.0	57	8.0	92	6.8	79	126	2.6	8.9
15 mg.	77-99*	11.0	62	682	5.6	62	7.9	87	6.6	73	124	2.5	8.5
S.C.	99-128	11.0	61	675	5.6	62	6.8	75	5.4	60	130	2.5	8.5
	128-156	6.1	82	498	7.9	48	7.0	42	6.8	41	113	1.8	4.3
15	25-45	18.2	61	1,100	14.1	258	5.0	91	11.9	218	138	4.0	14.2
15 mg.	45-65*	19.5	65	1,260	16.8	328	5.5	107	14.1	275	135	4.6	14.9
S.C.	65-85	18.8	66	1,240	17.2	324	6.9	129	14.1	264	128	4.5	14.3
	85-106	17.6	72	1,270	18.6	328	7.5	132	15.8	282	123	4.6	13.0
	106-130	18.0	79	1,430	23.3	423	7.5	136	20.2	365	126	5.2	12.8
	130-159	14.0	76	1,060	18.8	263	7.1	99	17.6	245	124	3.8	10.2
16	90-120	9.4	85	796	10.7	100	5.7	54	10.4	98	135	2.8	6.6
15 mg.	120-150*	10.0	83	830	13.0	130	4.9	49	11.7	117	132	2.9	7.1
S.C.	150-210	11.6	80	910	12.9	155	3.9	44	12.2	140	141	3.2	8.4

*Morphine administered at the beginning of this period except in Subject 15, to whom it was administered at the mid-point of the period.

TABLE III. EXPERIMENTS IN WHICH A LATE DECREASE IN URINE FLOW WAS ASSOCIATED WITH A RISE IN URINE OSMOLALITY

EXPERIMENT NO.	ELAPSED TIME (MIN.)	FLOW (ML./MIN.)	URINE								CLEARANCES		
			TOTAL SOLUTE		SODIUM		POTASSIUM		CHLORIDE		C_{Cr} (ML./MIN.)	C_{Osm} (ML./MIN.)	C_{H_2O} (ML./MIN.)
			mOSM./L.	μOSM/MIN.	MEQ./L.	μEQ/MIN.	MEQ./L.	μEQ/MIN.	MEQ./L.	μEQ/MIN.			
1b	41-61	22.2	61	1,350	12.4	274	7.4	165	15.8	349	150	4.8	17.4
	61-81*	20.4	61	1,240	11.8	241	7.4	151	14.8	301	144	4.5	15.9
	81-101	15.6	56	874	7.3	114	5.5	85	9.1	142	140	3.2	12.4
	101-131	13.4	57	764	7.7	103	4.6	62	8.4	113	129	2.7	10.7
	131-162	5.3	135	719	27.0	143	10.6	56	30.4	162	127	2.6	2.7
	162-192	5.2	178	926	37.7	196	11.2	58	40.9	213	138	3.4	1.8
	192-222	11.3	90	1,020	22.1	250	4.6	52	20.5	232	139	3.7	7.6
	222-242	14.4	73	832	19.1	275	4.0	57	17.1	246	141	3.1	11.3
	242-262	15.4	71	1,090	19.3	297	3.2	50	17.6	269	138	4.0	11.4
1c	40-60	20.9	50	1,040	8.4	176	5.7	120	10.3	214	141	3.8	17.1
	60-80*	18.9	50	943	10.0	189	6.3	118	11.8	223	137	3.4	15.5
	80-100	15.2	54	821	9.6	145	5.9	90	9.6	146	128	3.0	12.2
	100-125	8.1	82	663	11.2	91	8.1	65	13.7	111	124	2.4	5.7
	125-155	8.5	92	670	12.9	109	7.6	64	14.0	119	123	2.4	6.1
	155-185	8.4	95	790	16.3	137	7.3	62	17.1	144	124	2.9	5.5
	185-215	11.8	71	840	12.8	151	5.4	64	11.9	141	124	3.1	8.7
	215-235	12.5	70	805	13.4	154	5.0	58	12.9	148	122	2.9	9.6
	235-255	10.2	76	771	14.5	147	4.2	42	13.9	141	123	2.8	7.4
3	42-63	17.5	63	1,100	11.3	198	7.0	123	14.7	257	130	4.0	13.5
	63-85*	12.7	66	840	13.5	172	7.3	93	18.4	235	89	3.0	9.7
	85-112	11.5	68	781	12.8	147	7.4	85	18.1	208	88	2.8	8.7
	112-146	11.0	69	759	11.3	124	7.3	80	15.7	176	98	2.8	8.2
	146-185	8.6	87	750	13.7	118	6.4	55	18.6	160	109	2.7	5.9

5	40-60	19.7	49	965	5.9	116	6.9	136	6.2	123	143	3.5	16.2
	60-81*	17.8	50	896	6.1	109	7.0	125	6.1	108	140	3.2	14.6
	81-101	14.3	50	719	4.9	70	5.8	84	4.7	67	132	2.6	11.7
	101-152†	2.8	142	397	17.6	49	12.2	34	15.9	44	95	1.4	1.4
7	40-60	18.4	50	920	4.1	75	8.5	156	7.0	129	150	3.3	15.1
	60-80*	19.0	51	969	4.4	84	8.8	167	7.5	143	148	3.5	15.5
	80-100	16.1	52	837	4.2	67	9.2	148	7.0	112	138	3.0	13.1
	100-121	14.2	53	750	3.9	55	8.1	114	6.2	87	138	2.7	11.5
	121-171	10.1	58	620	4.9	52	7.8	83	5.7	61	134	2.3	7.8
	171-203	1.5	242	363	35.7	53	31.7	48	31.3	47	114	1.3	0.2
8	40-60	15.1	73	1,094	9.6	144	8.5	128	11.6	175	167‡	4.0	11.1
	60-80*	14.8	70	1,033	10.3	152	7.3	108	10.8	159	163	3.8	11.0
	80-100	12.8	73	921	9.0	113	7.3	92	9.6	121	157	3.4	9.4
	100-120	12.3	71	872	9.9	121	5.6	69	8.6	105	160	3.2	9.1
	120-140	10.9	80	869	11.9	130	5.6	61	9.3	102	160	3.2	7.7
	140-161	4.0	142	573	25.3	103	9.3	38	19.0	77	114	2.1	1.9
	161-202	1.3	390	501	78.0	101	24.2	31	53.8	70	114	1.8	−0.5
14	49-77	11.5	62	715	5.0	57	8.0	92	6.8	79	126	2.6	8.9
	77-99*	11.0	62	682	5.6	62	7.9	87	6.6	73	124	2.5	8.5
	99-128	11.0	61	675	5.6	62	6.8	75	5.4	60	130	2.5	8.5
	128-156	6.1	82	498	7.9	48	7.0	42	6.8	41	113	1.8	4.3

*Morphine administered at the beginning of this period except in Experiments 7 and 8, when it was given at the mid-point of the period.

†Experiment 5 was continued for 279 minutes and the urine osmolality increased to 550 mOsm./L., but data are not presented because of incomplete bladder emptying during that entire time.

‡Corresponding values for inulin clearance were 158, 158, 145, 150, 148, 104, and 107 ml./min. Simultaneous PAH clearances were 592, 598, 507, 528, 566, 383, and 400 ml./min.

rise in urinary concentration.* Experiment 9a (Fig. 3) is of particular interest in view of the fact that the initial decrease in diuresis was followed by a subsequent return toward the control values. These changes in urine flow were associated with a decline and later recovery of creatinine clearance and solute excretion. There was very little change in urinary concentration throughout the experiment.

3. In 5 studies a prompt reduction in urine flow greater than 2 ml./min. did not follow upon the administration of morphine (Table II). In Experiments 9b and 13 there was a decline in flow of 2.0 ml./min. and 1.7 ml./min., respectively, within 40 minutes after morphine was given, associated with a decrease in solute excretion, but without decrease in creatinine clearance. Urine osmolality rose slightly. Subjects 14 and 15 had a late fall (within 79 and 104 min. after morphine, respectively) in urine flow of more than 2 ml./min. with a decrease in the rate of solute excretion and a reduction in creatinine clearance. In both instances there was an over-all rise in urine osmolality of more than 10 mOsm./L. Subject 16 had a rise in urine flow, increased rate of solute excretion, and a small increase in creatinine clearance without significant change in concentration of the urine.

4. In 6 (Subjects 1b, 1c, 3, 5, 7 and 8) of the 17 experiments, in which the administration of morphine induced a prompt decline in urine flow without rise in osmolality (Table I), there was a later decrease in urine flow of more than 2 ml. per minute. This decrease was associated with an abrupt rise in urine osmolality of more than 10 mOsm./L. and occurred 65 to 143 minutes after morphine administration (Table III). In Experiments 1b and 1c these changes showed definite evidence of reverting toward the control values within 90 minutes. A similar late decrease in flow and rise in urine concentration took place in Experiment 14 in which the earlier decline did not occur (Table III). In 5 of these 7 instances, the patients experienced nausea.

DISCUSSION

The administration of morphine in therapeutic doses to normal men during water diuresis generally resulted in a decrease in the rate of urine flow which cannot be attributed to increased antidiuretic hormone activity in view of the fact that the concentration of the urine did not increase. Although a modest rise in osmolal concentration of a hypotonic urine may not be attributed to antidiuretic hormone effect with complete confidence, unless filtration rate remains the same or increases and the rate of solute excretion remains the same or decreases,[20-22] it is apparent that increased ADH activity cannot be invoked to explain a decrease in flow unless the urine becomes more concentrated. The administration of the alkaloid was followed by a decrease in the rate of solute excretion and reduction in filtration rate, changes that alone or together are sufficient to account for a decrease in the rate of urine flow.† In the 7 subjects

*The late marked fall in creatinine clearance in this experiment is probably at least in part an artifact due to "dead space error" during an abrupt fall in flow.[19]

†On the basis of recent observations in dogs[20, 21] it might be expected that an abrupt fall in filtration rate alone, without the intervention of ADH, would lead to an increase in urine osmolality. Such an association in the present study was not observed even when endogenous creatinine clearance was decreased by over 30 per cent. It is apparent that a species difference may exist. Moreover, even in the dog increased urine concentration in response to lowered filtration rate has not always been observed.[23] Precise definition of factors determining this relationship requires further elucidation.

who demonstrated a late rise in urine osmolality, it is probable that a subsequent release of ADH occurred. It may be noted that 5 of these subjects had nausea which in itself might stimulate secretion of ADH. Since the action of ADH is almost instantaneous, the delayed antidiuretic response cannot be ascribed to an earlier release of hormone.[24]

The data derived from extensive animal studies indicate that under certain conditions morphine acts to stimulate the release of ADH. DeBodo[2] observed unequivocal inhibition of water diuresis in normal dogs who received 2.5 to 5.0 mg. per kilogram of body weight of morphine sulfate subcutaneously or intravenously. Further, he showed that an intact neurohypophyseal system was necessary for the antidiuretic action, and that morphine inhibited diuresis even after one adrenal gland was removed while the other was denervated and demedullated. He also demonstrated that morphine did not potentiate the action of posterior pituitary extract in dogs with diabetes insipidus. Duke, Pickford, and Watt[25] observed that very much smaller intravenous doses of morphine (0.08 mg. per kilogram or less) produced antidiuresis in the dog.[25] This response was abolished by section of the supraopticohypophyseal tract. The final evidence for a direct effect of morphine on the supraopticohypophyseal system under these conditions was provided by the same investigators who demonstrated antidiuresis after 4 to 32 μg of morphine were injected into one or both supraoptic nuclei during water diuresis in chloralose anesthetized dogs. Other investigators have reported that the antidiuresis induced by morphine in animals with intact posterior pituitary gland was associated with the appearance, in the urine, of an antidiuretic material which was not morphine and which resembled vasopressin.[3, 5-7] Nallorphine, which had no effect on diuresis in rats, has been reported to inhibit the antidiuretic action of morphine.*[6, 7] It would thus seem that in the dog the administration of morphine stimulates the release of ADH and it also seems fairly clear that in large doses it has the same effect in the rat.

The possible role of altered renal hemodynamics in the decline in urine flow following morphine administration in animals has also received some attention. Antidiuretic action of the drug has been reported in dogs with diabetes insipidus.[4, 9] Handley and Keller concluded that a dose of 1 mg. of morphine per kilogram intravenously in the dog acted only to stimulate ADH while 2 mg. per kilogram also resulted in a decrease in filtration rate (creatinine clearance).[4] Crawford and Pinkham[8] also postulated a dual mechanism of morphine action; a "potentiation" of ADH and a renal mechanism, the latter predominant in "hyposthenuric" rats. As indicated above, Duke, Pickford, and Watt[25] observed inhibition of water diuresis by very small doses of morphine unrelated to changes in renal plasma flow or filtration rate.

*Our observations concerning nallorphine are limited to one experiment. Subject 5 was given 15 mg. of the drug subcutaneously on one occasion. His urine flow decreased within 20 minutes from 19.6 ml./min. to 13.4 ml./min. without change in urine osmolality (47 and 48 mOsm./L.), but with a decline in creatinine clearance from 120 ml./min. to 98 ml./min. and a reduction in solute excretion from 925 μOsm/min. to 634 μOsm/min. Forty minutes after nallorphine administration he became extremely nauseated and experienced bizarre feelings that he was unable to describe.

A variety of diverse experiments in human subjects have been reported. The administration of morphine sulfate in therapeutic doses has been reported to cause a reduction in urine flow by a number of investigators.[10-12, 14] In some instances this has been shown to be associated with a rise in urinary concentration of sodium and chloride[10, 14] and a reduction in filtration rate.[14] In one study there was an accompanying decline in paraaminohippurate clearance without change in filtration rate as determined by the clearance of sodium thiosulfate.[12] The study of Kraushaar, Bradbury, Wang, and Brown[11] is of interest in that morphine inhibited the rate of diuresis without increase in urinary specific gravity or urinary chloride concentration. The rate of chloride excretion generally decreased. The same investigators also observed that the administration of morphine to 2 women considered to have diabetes insipidus was followed by a decrease in diuresis, albeit less striking than in the normal. They concluded that increased ADH activity cannot explain the effect of morphine on the rate of urine flow in normal women. DeBodo, commenting on these observations, was of the opinion that the studies did not constitute evidence against increased ADH as the mechanism of the morphine induced antidiuresis and suggested that the 2 women with diabetes insipidus had some residual posterior pituitary tissue.[26] Walker, on the other hand, gave 20 mg. of morphine subcutaneously to 7 normal subjects and observed no change in urine flow despite the presence of nausea and vomiting in some instances.[13] None of these studies in man included measurements of total osmolal concentration of the urine nor was there demonstrated a rise in solute concentration without change in filtration rate. Thus the evidence for ADH release in man following morphine administration is limited.

In contrast to the demonstrated stimulation of ADH release in animals given morphine, it is apparent that in normal man morphine can decrease the magnitude of a water diuresis independent of increased ADH activity. This difference may be attributed to species variation or to the large doses of morphine employed in most of the animal studies compared with the smaller quantities administered to man. However, the latter explanation cannot apply to the observations of Duke, Pickford, and Watt who found evidence for ADH release in dogs after the intravenous administration of morphine in doses as low as 0.08 mg. per kilogram intravenously.[25]

The mechanism responsible for the observed reduction of creatinine clearance and solute excretion after morphine administration cannot be inferred from the data presented. Morphine has little effect on cardiac output, blood pressure, or peripheral resistance in normal recumbent man, although orthostatic hypotension is reported.[27, 28] Blood pressure did not change in our studies. Therefore, it is possible that morphine alters renal function by a direct effect on the intrarenal circulation.

The duration of the alteration in urine flow, solute excretion and creatinine clearance was variable. In one half of the subjects who exhibited a decline in creatinine clearance after morphine, a return toward the control values occurred from 70 to 218 minutes after alkaloid administration, while in the other half

no such trend was observed during the experimental period. Urine flow did not return to control values during the period of observation, but the role of late ADH release in 7 experiments and the superimposed influence of the diurnal excretory rhythm of water and solute complicates interpretation.

The present study was not designed to determine the ability of patients, whose clinical management requires the frequent administration of morphine, to excrete a large volume of administered water during a 24-hour period. However, it is apparent that the reduction in urine flow induced by a single dose of morphine in a majority of subjects, would not have compromised their ability to excrete copious urine during a period of several hours. More marked impairment was observed only in those instances when pronounced ADH effect was manifest. On the other hand, it is possible that in patients who are experiencing pain with consequent ADH release, the administration of morphine by allaying pain may inhibit ADH release and hence promote diuresis.*

CONCLUSIONS

1. Morphine sulfate was administered in therapeutic doses, subcutaneously and intravenously, on 22 occasions to 16 normal men undergoing water diuresis.

2. In 17 of the 22 experiments, the administration of morphine was followed within 10 to 55 minutes by a decrease in urine flow of more than 2.0 ml. per minute. The decrease in flow was *unaccompanied* by a rise in urine osmolality, but was associated with a decrease in endogenous creatinine clearance and the rate of total solute excretion.

3. In 7 experiments, there was a late decrease in urine flow associated with a rise in urine osmolality.

4. Morphine sulfate, administered in therapeutic doses to normal men, may result in a decrease in urine flow without evident release of antidiuretic hormone. The decrease in diuresis may be attributed rather to the observed decrease in glomerular filtration rate and in rate of solute excretion.

REFERENCES

1. Heller, H.: Antidiuretic Substances, J. Pharm. & Pharmacol. **3**: 609, 1951.
2. DeBodo, R. C.: The Antidiuretic Action of Morphine and Its Mechanism, J. Pharmacol. & Exper. Therap. **82**: 74, 1944.
3. Lipschitz, W. L., and Stokey, E.: Mechanisms of Antidiuresis in the Dog and in the Rat, Am. J. Physiol. **148**: 259, 1947.
4. Handley, C. A., and Keller, A. D.: Changes in Renal Function Produced by Morphine in Normal Dogs and Dogs With Diabetes Insipidus, J. Pharmacol. & Exper. Therap. **99**: 33, 1950.
5. Giarman, N. J., Mattie, L. R., and Stephenson, W. F.: Studies on the Antidiuretic Action of Morphine, Science **117**: 225, 1953.
6. Giarman, N. J., and Condouris, G. A.: The Antidiuretic Action of Morphine and Some of Its Analogs, Arch. internat. pharmacodyn. **97**: 28, 1954.

*One subject was studied under conditions of a single unsustained oral water load. After his flow had decreased gradually from a maximum of 20.8 ml./min. with an associated osmolality of 57 mOsm./L., to a flow of 3.5 ml./min. and an osmolality of 307 mOsm./L., 15 mg. of morphine sulfate were given subcutaneously. Within the next 20 minutes there was a further decrease in flow to 1.9 ml./min. and a rise in osmolality to 455 mOsm./L. However in the subsequent collection period, within 55 minutes after morphine, urine flow was 2.4 ml./min. with a concentration that had decreased to 348 mOsm./L. despite an associated decline in creatinine clearance of 13 ml./min. In the following periods the urine once again became more concentrated. Although the only symptoms presented were related to morphine administration ("lightheadedness" and unsteadiness) it is possible that the sedative action of the drug resulted in some *inhibition* of ADH release.

7. Winter, C. A., Gaffney, C. E., and Flataker, L.: The Effect of N-allylnormorphine upon the Antidiuretic Action of Morphine, J. Pharmacol. & Exper. Therap. **111:** 360, 1954.
8. Crawford, J. D., and Pinkham, B.: The Physiology of Morphine Antidiuresis, J. Pharmacol. & Exper. Therap. **113:** 431, 1955.
9. George, R., and Way, E. L.: Morphine Antidiuresis in Rats With Hypothalamic Lesions, Fed. Proc. **15:** 427, 1956.
10. Ferrer, M. I., and Sokoloff, L.: The Antidiuretic Effect of Morphine and Demerol in Congestive Heart Failure, Am. J. M. Sc. **214:** 372, 1947.
11. Kraushaar, O. F., Bradbury, J. T., Wang, Y. K., and Brown, W. E.: Morphine Suppression of Urinary Output in Pregnant and Nonpregnant Women, Am. J. Obst. & Gynec. **57:** 302, 1949.
12. Brown, W. E., Hodges, R., and Bradbury, J. T.: Effects of Morphine on Renal Clearances of Para-Amino Hippurate and Sodium Thiosulfate in the Human Kidney, Am. J. Med. **6:** 663, 1949.
13. Walker, J. M.: The Effect of Smoking on Water Diuresis in Man, Quart. J. Med. **18:** 51, 1949.
14. Habif, D. V., Papper, E. M., Fitzpatrick, H. F., Lowrance, P., Smythe, C. McC., and Bradley, S. E.: The Renal and Hepatic Blood Flow, Glomerular Filtration Rate, and Urinary Output of Electrolytes During Cyclopropane, Ether, and Thiopental Anesthesia, Operation, and the Immediate Postoperative Period, Surgery **30:** 241, 1951.
15. Strauss, M. B., Davis, R. K., Rosenbaum, J. D., and Rossmeisl, E. C.: "Water Diuresis" Produced During Recumbency by the Intravenous Infusion of Isotonic Saline Solution, J. Clin. Invest. **30:** 862, 1951.
16. Young, M. K., Jr., and Raisz, L. G.: An Anthrone Procedure for Determination of Inulin in Biological Fluids, Proc. Soc. Exper. Biol. & Med. **80:** 771, 1952.
17. Goldring, W., and Chasis, H.: Hypertension and Hypertensive Disease, The Commonwealth Fund, London, 1944, Oxford University Press, p. 203.
18. Smith, H. W.: Renal Excretion of Sodium and Water, Fed. Proc. **11:** 701, 1952.
19. Smith, H. W.: The Kidney. Structure and Function in Health and Disease, New York, 1951, Oxford University Press, p. 61.
20. Del Greco, F., and de Wardener, H. E.: The Effect on Urine Osmolarity of a Transient Reduction in Glomerular Filtration Rate and Solute Output During a "Water" Diuresis, J. Physiol. **131:** 307, 1956.
21. Berliner, R. W., and Davidson, D. G.: Production of Hypertonic Urine in Absence of Pituitary Antidiuretic Hormone, J. Clin. Invest. **35:** 690, 1956.
22. de Wardener, H. E., and Del Greco, F.: The Influence of Solute Excretion Rate on the Production of a Hypotonic Urine in Man, Clin. Sc. **14:** 715, 1955.
23. Leaf, A., Kerr, W. S., Jr., Wrong, O., and Chatillon, J. Y.: Effect of Graded Compression of the Renal Artery on Water and Solute Excretion, Am. J. Physiol. **179:** 191, 1954.
24. Verney, E. B.: Antidiuretic Hormone and the Factors Which Determine Its Release, Proc. Roy. Soc., London, s.B. **135:** 25, 1947.
25. Duke, H. N., Pickford, M., and Watt, J. A.: The Antidiuretic Action of Morphine: Its Site and Mode of Action in the Hypothalamus of the Dog, Quart. J. Exper. Physiol. **36:** 149, 1951.
26. DeBodo, R. C.: Comments on the Antidiuretic Action of Morphine, Am. J. Obst. & Gynec. **58:** 1825, 1949.
27. Papper, E. M., and Bradley, S. E.: Hemodynamic Effects of Intravenous Morphine and Pentothal Sodium, J. Pharmacol. & Exper. Therap. **74:** 319, 1942.
28. Drew, J. H., Dripps, R. D., and Comroe, J. H., Jr.: Clinical Studies on Morphine. II. The Effect of Morphine Upon the Circulation of Man and Upon the Circulatory and Respiratory Responses to Tilting, Anesthesiology **7:** 44, 1946.

COMPLEMENT-FIXING ANTIBODY RESPONSE TO M-PROTEIN OF NEPHRITOGENIC STREPTOCOCCI IN GLOMERULONEPHRITIS

MARY BONE, M.D.,* ABRAHAM I. BRAUDE, M.D., AND HERMAN KLEINMAN, M.D.
DALLAS, TEXAS

THE predominant isolation of type 12 streptococci from patients with acute glomerulonephritis has led to the hypothesis that only certain streptococcal types have nephritogenic properties.[1] If this hypothesis is valid, patients with glomerulonephritis should develop type-specific antibodies to streptococcal M-protein of nephritogenic types. In an effort to detect such antibodies, a series of nephritic sera were examined by the mouse protection and bacteriostatic methods. Although the results clearly demonstrated the occurrence of type-specific antibody to nephritogenic streptococci in the sera, it was apparent that technical and biologic factors limited the application of these tests. The protection test required not only a mouse-virulent streptococcus, but also large quantities of serum; and the bacteriostatic test was not always reliable if acute-phase substance or antibioties were present in the patient's sera. Because these difficulties could be circumvented with a procedure that did not require living streptococci a complement fixation test was devised using M-protein of nephritogenic streptococci as the antigen. An extensive search of the literature revealed no previous report demonstrating complement-fixing antibodies to streptococcal M-protein in human sera.

MATERIALS AND METHODS

Clinical Material.—The complement fixation test was applied to sera from 3 separate groups. The first consisted of 54 Indian children from Red Lake, Minnesota, with an average age of 5 years, 44 per cent of whom were boys. Serum was obtained from these patients 2 years after their recovery from acute epidemic glomerulonephritis occurring in the Red Lake Indian Reservation of Minnesota.[2] The second group was composed of 52 cases of glomerulonephritis which occurred sporadically in the Dallas area. The average age for this group was 15 years, with 53 per cent of the cases occurring in males. Serum was obtained during the acute illness and for periods up to 36 months following recovery. The final group consisted of 52 nonnephritic adults who served as normal controls.

The diagnosis of acute glomerulonephritis in the patients of group II was suggested in all cases by hematuria. The majority also developed edema, hypertension, a rising ASO titer, or a combination of these features which established the diagnosis beyond a doubt.

Antistreptolysin O.—All ASO titers were determined by the procedure of Rantz and Randall.[3]

From the Department of Internal Medicine, Southwestern Medical School, Dallas, Texas and the Red Lake Indian Reservation, Red Lake, Minn.

Supported in part by a U. S. Public Health Service research grant.
Received for publication June 3, 1957.

*Public Health Service Research Fellow of the National Institutes of Health.

Antigens.—The antigens were prepared by the acid-extraction method of Lancefield[4] from 4 types of Group A streptococci: 3, 40, 12, and the new provisional Red Lake (RL) type.[5] All organisms were typed as such in the laboratories of Dr. Elaine Updyke at the Communicable Disease Center in Chamblee, Georgia. The RL and type 12 streptococci were isolated from patients with acute glomerulonephritis, the RL from a patient in Dallas. The cell sediment from an 18-hour growth in 4.5 L. of Todd-Hewitt broth was obtained by centrifugation, resuspended in 100 ml. N/20 HCl and heated in a boiling water bath for 10 minutes. After cooling, the suspension was neutralized with 1 N NaOH to a pH of 7.2 to 7.4. The clear, yellowish supernatant obtained by centrifugation of the neutralized suspension was stored at 3° to 6° C. after Merthiolate was added to a final concentration of 1:20,000.* Antigenic activity of these extracts remained stable during refrigeration for a period of 4 or 5 months. The extracts were discarded when clouding or contamination rendered them unsatisfactory for use in the complement fixation test.

Antigenic titration was performed with varying dilutions of the extract against a 1:10 dilution of absorbed sera obtained from two patients with acute glomerulonephritis, one following a type 12 and the other a RL streptococcal infection. The type 12 antigen was also titrated against a 1:10 dilution of absorbed rabbit antiserum prepared by the method outlined in *Standard Methods.*[6] The antigenic unit was the smallest amount of antigen preventing hemolysis of sensitized sheep cells in the presence of a positive human serum, 2 full units of complement, and 2 full units of hemolysin.† Two antigenic units were used in the complement fixation test as recommended by Kolmer[7] whose procedure was followed throughout. Because no positive sera were avaibable for titration of the T3 and T40 antigens, undiluted extracts were used in the tests.

Sera.—All sera were absorbed with a streptococcus of a type different than those from which the M-protein antigens were extracted in order to eliminate nonspecific reactions with traces of other antigens which might not have been removed during the acid extraction procedure. Absorption was performed by mixing equal parts of serum and a suspension of packed cells of heat-killed T2 streptococci, usually in the quantity of 1 ml. each. The cells which had been killed by heating for one hour at 56° C. were mixed thoroughly with the serum and incubated at 37° C. for 30 minutes, followed by 18 hours at 3° C.[8] The serum was separated from the cells by centrifugation (5,000 to 6,000 r.p.m.) for one hour. The supernatant serum was transferred to clean test tubes and recentrifuged at the same speed for 30 minutes; this procedure was repeated and the serum then inactivated by heating for 30 minutes in a 56° C. water bath. The repeated rapid centrifugation and complete removal of all absorbing cells effectively avoided the reported tendency for absorption to render serum anticomplementary.[9]

Technique of Complement Fixation Test.—A quantitative complement fixation test was performed according to the method of Kolmer.[10] Since identical results were obtained with the regular and one fifth quantitative methods, the latter has been used more often to conserve serum. Tests were considered negative when complete hemolysis was noted in all tubes; doubtful reactions were recorded when the first tube (1:4 serum dilution) contained a questionable or 1 + turbidity; and a positive test contained at least a 2 + turbidity in the first tube.

RESULTS

Epidemic Nephritis.—Among the unabsorbed sera obtained from the 54 Red Lake Indian children 2 years after the epidemic of glomerulonephritis, 53 fixed complement with a RL extract and 33 did so with the T12 extract. After absorption of 33 of the sera with the indifferent streptococcus, T2, 19 remained

*The addition of Merthiolate may raise the pH slightly and neutralization may be required with 1 N HCl.

†Dried guinea pig complement obtained commercially from Hyland Laboratories and Bacto-Antisheep hemolysin from Difco Laboratories were used in all tests after first titrating the exact amount to be contained in two full units.

positive and 7 doubtful with the RL antigen; whereas 2 were positive and 2 doubtful with the T12 antigen (Table I). The prolonged antibody response in these Indians at Red Lake may be related to the persistence of the RL streptococcus in the epidemic area and the repeated occurrence of subclinical exposures during the ensuing 2 years. This is supported by the isolation of the Red Lake streptococcus from the nasopharynx of asymptomatic carriers during cultural studies performed at the time the RL sera were obtained. It is also possible that antibiotic therapy may have been insufficient to eradicate the etiologic agent since the average total penicillin dose of 1.3 million units received by the RL nephritic was only 1/10 that given to the average sporadic nephritic in the Dallas area.

TABLE I. RESULTS OF C. F. TEST USING UNABSORBED AND ABSORBED SERA FROM 33 RED LAKE INDIANS TWO YEARS AFTER THEIR RECOVERY FROM ACUTE GLOMERULONEPHRITIS

PATIENT	ASO	RED LAKE ANTIGEN		TYPE 12 ANTIGEN	
		UNABSORBED	ABSORBED SERA	UNABSORBED	ABSORBED SERA
A. S.	250	444300*	43±000	442000	–
W. S.	125	444000	–	±	–
M. A. G.		432000	–	444200	41±000
L. G.	333	444400	3±0000	444430	–
P. G.	166	444000	±	421000	–
G. H.	333	4444±0	300000	432100	–
J. M.	333	444400	300000	444100	–
E. M.	125	444400	400000	3±0000	–
L. D.	50	442±00	±	–	–
A. D.		444000	–	–	–
J. G.	100	442000	442000	441000	400000
S. D.	250	442000	–	–	–
E. S.	166	444300	411000	–	–
B. W.	166	43±000	–	–	–
M. H.	250	441±00	–	–	–
B. R.	100	444400	4±1000	22±000	–
M. H.	500	444400	3±0000	–	–
D. D.		442000	400000	±	±
W. G.	100	443000	411000	–	–
H. L.	250	444100	4±0000	443000	–
B. S.	166	440000	±	200000	–
H. S.	100	444100	±	444300	–
J. S.	166	444000	320000	4±0000	–
J. K.	333	444400	±	411000	–
A. K.	166	444100	±	41±000	–
J. R. H.	333	444400	441000	433100	±
J. A. L.	250	444300	400000	±	–
V. G.	166	442000	–	400000	–
J. G.	100	443±00	4±0000	444100	–
W. G.	333	444400	443000	444200	–
R. G.	100	444000	±	–	–
D. H.	100	444400	410000	444400	–
F. D.	100	444200	430000	41±000	–

*1, 2, 3, 4, = 1+, 2+, 3+, or 4+ complement fixation; the series of numbers represents the reactions in serial dilutions of sera beginning with 1:4.

Sporadic Nephritis.—Unlike the Red Lake sera, the absorbed sera from the 52 Dallas nephritics reacted as frequently with the T12 antigen as with the RL antigen (Table II). Eleven sera gave a positive reaction when tested with either antigen and 13 sera which were positive with one antigen gave a doubtful

TABLE II. RESULTS OF C. F. TEST USING ABSORBED SERA FROM 52 DALLAS NEPHRITICS

PATIENTS	ASO	DAYS AFTER ONSET OF NEPHRITIS	RED LAKE ANTIGEN	TYPE 12 ANTIGEN
E. P.	500	10	3±0000	442000
E. B.	50	425	2±0000	±
M. S.	166	16	200000	42±000
O. S.	333	540	±	-
W. C. A.	100	995	-	-
C. V.	625	60	441000	-
A. C.	650	31	4±0000	±
J. F.	125	100	43±000	±
D. J.	500	9	3±0000	420000
O. D.	1,250	28	300000	440000
H. W.	333	14	±	±
M. B.	625	22	±	±
L. C.	333	940	-	-
P. A.	333	26	-	-
R. L. T.	100	540	-	213000
F. R.	125	510	±	210000
J. M.	125	11	±	±
S. W.	125	300	-	-
J. J.	333	10	-	-
E. J.	500	17	-	-
P. R.	333	6	200000	400001
L. R.	625	6	4±0000	±
F. R.	100	58	200000	4±0000
J. W.	250	5	-	-
J. D.	166	21	-	-
D. D.	166	14	±	-
S. L.	625	20	±	-
B. H.	333	14	-	-
C. S.	333	32	±	±
V. B.	125	21	-	-
F. H.	833	18	4±0000	210000
O. M. E.	333	23	±	42±001
M. F.	2,500	18	43±000	444400
J. S.	500	46	-	-
L. H.	250	44	-	-
J. R.	625	18	444300	444440
E. R.	625	6	±	421000
B. C.	333	18	-	300000
J. M.	2,500	29	444100	444440
J. D.	500	87	±	-
J. D. C.	333	28	±	200000
O. M.	333	14	200000	400000
L. W.	125	6	±	±
M. S.	100	14	-	-
K. S.	250	90	-	-
W. B.	250	109	-	-
B. R. L.	625	57	±	200000
A. P.	333	90	2±0000	-
S. F.	250	51	±	200000
L. L.	250	15	-	-
J. W.	250	12	±	200000
F. S.	625	21	-	420000

reaction to the heterologous extract. This marked overlap in reactivity obtained with these two antigens led to the speculation that a common antigenic substance may be shared by those streptococci having nephritogenic properties. In order to decide which of the two streptococcal types initiated the infection serial tests were done in 5 patients over a period varying from 2 weeks to a year and a comparison was made of the duration and titer of the antibody

response to the 2 nephritogenic streptococci in a given patient (Table III). The results demonstrated that one type-specific antibody consistently predominated in the sera of a given patient over a period of time. The streptococcus to which this dominant antibody corresponded was regarded as the etiologic organism.

TABLE III. SERIAL C. F. TESTS USING ABSORBED SERA FROM 5 DALLAS NEPHRITICS

NAME AND SERUM DATE	ONSET DATE AND ASO TITER	TYPE 12 ANTIGEN	RED LAKE ANTIGEN	REMARKS[11]
E. P.	9-3-55			
10- 3-55	250	432000	3±0000	Mouse protective and bacteriostatic antibodies to type 12
10-13-55	500	442000	3±0000	
10-26-55	250	42±000	3±0000	
11- 4-55	333	42±000	100000	
12-14-55	333	410000	310000	
4-25-56	166	210000	200000	
7-11-56	250	210000	100000	
10-10-56	250	110000	100000	
M. S.	12-14-56			
12-21-56	166	3±1000	±00000	Mouse protective and bacteriostatic antibodies to type 12
12-27-56	166	3±0000	100000	
12-29-56		42±000	200000	
1- 4-57	250	420000	100000	
C. V.	2-3-55			Red Lake streptococcus isolated from throat
3-21-55	333	000000	441000	
4-13-55	625	000000	44±000	
4-21-55	166	000000	4±0000	Mouse protective and bacteriostatic antibodies to RL
D. J.	2-9-56			
2-16-56	500	420000	3±0000	Mouse protective and bacteriostatic antibodies to type 12
3- 3-56	333	444000	±00000	
3-12-56	625	441000	100000	
7-18-56	250	±00000	±00000	
O. D.	6-19-56			Mouse protective and bacteriostatic antibodies to type 12
7-11-56	625	410000	000000	
7-18-56	625	420000	200000	
7-25-56	1,250	440000	300000	

TABLE IV. RESULTS OF C. F. TEST USING SERUM FROM A DALLAS NEPHRITIC ABSORBED WITH BOTH T2 AND T12 STREPTOCOCCI

(Serum was obtained 6 weeks after onset of sore throat when ASO titer was 625)

PATIENT J. R.	TYPE 12 ANTIGEN	RED LAKE ANTIGEN
Serum Absorbed with T2	444440	444300
Serum Absorbed with T12	000000	430000

The cross reactivity between the two antigens with a given serum raised the possibility that the RL and the T12 antigens might be the same rather than related. The individuality of the two antigens was established by demonstrating the persistence of the RL antibody after removal of T12 antibody by absorption with T12 streptococcal cells (Table IV).

Reactions With T3 and T40 Extracts.—Absorbed sera from nephritics were tested with M-antigens of T3 and T40 streptococci. Of the 21 sera tested with T3, one was positive (1:4) and two doubtful. All 3 sera which showed this weak reactivity with the T3 antigen were strongly positive when tested with the T12 antigen. Twelve nephritic sera tested with the T40 antigen all gave negative results.

Normal Persons.—Among sera from 52 nonnephritics, 18 of whom had ASO titers ranging from 250 to 1,250, 2 contained antibody against the RL antigen, and only one against T12. The titers of the positives were only 1:4. There was no overlap in reactivity among the positive sera.

DISCUSSION

The concept that acute glomerulonephritis is a result of streptococcal infection has recently evolved to the point where only a limited number of streptococcal types are considered to possess nephritogenic potentialities. The evidence in favor of the existence of only a few types of nephritogenic streptococci is based mainly on isolation of these streptococci from patients, and suffers from the limitation that in nonepidemic areas cultures of many patients with nephritis are either negative or yield nontypable streptococci. Among 40 Dallas cases of sporadic glomerulonephritis in this study, for example, streptococci could be isolated from only 20; and of the 14 subjected to typing studies, 10 were nontypable, 3 were T12, and 1 was type RL. The complement fixation test used in this study has been of value, therefore, not only in providing further evidence of infection by those streptococci which have been implicated in glomerulonephritis, but also in extending it by serologic means to include the culturally negative nephritics. While the main objective in this investigation has been to provide a serologic method for obtaining such evidence, the complement fixation test can also be used to help identify those cases in whom the diagnosis of streptococcal nephritis is not clear on clinical grounds. There is a striking lack of type-specific antibody to types 12 and RL in nonnephritics with high ASO titers despite the fact that 12 predominates among the typable streptococci isolated from streptococcal infections occurring in nonnephritics in Dallas.

This difference in type-specific antibody titer in the sera of nephritics and nonnephritics might represent a greater tendency for nephritics to react immunologically to the M-antigens, but more extensive study would be needed to answer this question. Of greater immunologic importance, however, is the finding that nephritics apparently possess antibody to the M-protein of more than one nephritogenic type. This would imply an immunologic relationship between the nephritogenic M-substances which can be demonstrated with immune sera of infected persons but not of animals inoculated with dead streptococci. It remains to be determined whether or not this serologic difference depends on an antigenic response unique to infection or to species differences (i.e., man versus animal), but it is evident that a common property exists which could account for the ability of certain streptococci of different types to

induce the same clinical disturbance. Because this common feature is antigenic, the possibility exists, therefore, that the mechanism of renal injury involves an immunologic process such as hypersensitivity.

CONCLUSIONS

1. A complement fixation test has been devised for detecting type-specific antibodies to streptococcal M-protein.

2. The results obtained by application of this test to sera from the Red Lake Indian children indicates that the Red Lake streptococcus is a separate nephritogenic type, immunologically different from type 12, is widely distributed geographically, and evokes a prolonged immunologic response which provides evidence by complement fixation of past nephritogenic infection.

3. The data obtained by application of this test to the Dallas nephritics suggest that nephritogenic streptococci contain a related antigenic substance other than the conventionally measured type-specific substance, which promotes a prolonged, but not indefinite, antibody response.

4. The lack of reactivity noted when normal sera with high ASO titers were tested with the type 12 and Red Lake antigens supports the specificity of the test.

5. The paucity of positive reactions obtained by testing the nephritic sera with the T3 and T40 extracts provided further evidence supporting the type specificity of the C. F. test.

6. The nephritics whose sera gave negative results with both T12 and RL antigens may have had an infection with one of the other so-called nephritogenic types not included in this study.

REFERENCES

1. Rammelkamp, C. H., Jr., and Weaver, R. S.: Acute Glomerulonephritis. The Significance of the Variations in the Incidence of the Disease, J. Clin. Invest. **32:** 345-358, 1953.
2. Kleinman, Herman: Epidemic Acute Glomerulonephritis at Red Lake, Minnesota Med. **37:** 479-483, 489, 1954.
3. Rantz, L. A., and Randall, Elizabeth: A Modification of the Technic for Determination of the Antistreptolysin Titer, Proc. Soc. Exper. Biol. & Med. **59:** 22-25, 1945.
4. Lancefield, Rebecca: The Antigenic Complex of Streptococcus Haemolyticus. II. Chemical and Immunological Properties of the Protein Fractions, J. Exper. Med. **47:** 469-480, 1928.
5. Updyke, E. L., Moore, M. S., and Conroy, Elizabeth: Provisional New Type of Group A Streptococci Associated With Nephritis, Science **121:** 171-172, 1955.
6. Wadsworth, A. B.: Standard Methods, Baltimore, 1947, Williams & Wilkins Company, p. 796.
7. Kolmer, J. A., Spaulding, E. H., and Robinson, H. W.: Approved Laboratory Technic, New York, 1951, Appleton-Century-Crofts, Inc., pp. 843-846.
8. Wadsworth, A. B.: Standard Methods, Baltimore, 1947, Williams & Wilkins Company, pp. 778-779.
9. Boyd, W. C.: Fundamentals of Immunology, New York, 1947, Interscience Publishers, p. 298.
10. Kolmer, J. A., Spaulding, E. H., and Robinson, H. W.: Approved Laboratory Technic, New York, 1951, Appleton-Century-Crofts, Inc. pp. 814-830.
11. Braude, A. I., Bone, M., Waggoner, B., and Hatton, B.: Type Specific Antibody Response to M-Protein of Nephritogenic Streptococci in Glomerulonephritis, J. Clin. Invest. **36:** 876, 1957.

AGENTS ISOLATED FROM PATIENTS WITH BOSTON EXANTHEM DISEASE DURING 1954 IN PITTSBURGH

FRANKLIN A. NEVA, M.D.,* AND SAVERIO M. ZUFFANTE, M.S.
PITTSBURGH, PA.

AGENTS cytopathogenic for tissue cultures were recovered from feces of patients with an unusual exanthematous disease which occurred during 1951 in Boston.[1] More recently another outbreak of a similar illness, for which the term Boston exanthem disease has been proposed, occurred in Pittsburgh during 1954.[2] The disease was observed as a sharp localized outbreak in a suburban community, and also in scattered cases from 4 different areas of the city. Agents cytopathogenic for tissue cultures were isolated from specimens of 12 patients in the community outbreak and from 6 of the scattered cases.

This paper presents additional laboratory studies, not previously reported, which are pertinent to the recovery of these agents from exanthem patients in Pittsburgh, with reference to optimum specimens and tissues for their primary isolation, and tests for neutralizing antibody. Also presented are results from attempts to isolate similar agents from patients with other illnesses, and the findings of a survey for neutralizing antibodies.

MATERIALS AND METHODS

Stool specimens were stored in the refrigerator for 1 to 7 days, or frozen at −15° C. for periods of several weeks to 6 months before preparation as 10 per cent suspensions in phosphate buffered saline. The suspensions were clarified by centrifugation at 8,000 to 10,000 r.p.m. for 30 minutes in the cold. Throat and mouth washings were collected in modified medium 199[3] and neutralized with $NaHCO_3$. Blood clots were ground as 50 per cent suspensions with sterile alundum in phosphate buffered saline within 24 hours after blood was drawn. Pharyngeal washings and suspensions of blood clot were centrifuged at 2,000 r.p.m. for 20 minutes at room temperature. All suspensions used as inocula for tissue cultures contained 200 to 500 mcg. and units per milliliter of streptomycin and penicillin, respectively, and were stored in glass sealed ampules in the dry ice chest if not tested immediately.

Two types of roller tube cultures from tissues of either rhesus or cynomolgus monkeys were used primarily†: monolayer cultures[4] of trypsinized kidney, and plasma clot cultures of testicular tissue. Kidney cultures were maintained on medium 199 with 2 per cent inactivated horse serum. A mixture of equal volumes of 199 and commercial bovine amniotic fluid containing 2 per cent inactivated horse serum and 0.1 mg. per milliliter soybean trypsin inhibitor constituted the medium for testicular cultures. All culture media contained 100 mcg. and 100 units per milliliter of streptomycin and penicillin, respectively. Kidney cultures were changed to fresh medium at 3- to 5-day intervals, while testicular cultures were changed every 5 to 7 days. For the few experiments in which human kidney tissue‡ was employed, the roller cultures were prepared in the same manner as monkey kidney.

From the Virus Research Laboratory, School of Medicine, University of Pittsburgh.
Aided by a grant from The National Foundation for Infantile Paralysis, Inc.
Received for publication June 10, 1957.
*Present address is The Department of Tropical Public Health, Harvard School of Public Health, Boston 15, Mass.
†The assistance of many members of the Virus Research Laboratory staff in preparing cell suspensions and furnishing monkey testes is gratefully acknowleged.
‡The cooperation of Dr. Anthony Susan and staff of the Children's Hospital of Pittsburgh made available surgical specimens of tissue.

All serum samples from suspected exanthem patients were stored at −15° C. Many sera from other individuals which were screened for antibody had been stored at +5° C. For neutralization tests all sera, either undiluted or after 1: 2 dilution with phosphate buffered saline, were inactivated at 56° C. for 30 minutes.

A fixed dilution of virus was added to twofold dilutions of serum so that the final inoculum of the mixture contained a calculated 10 to 100 tissue culture infective dose ($TCID_{50}$). Serum-virus mixtures were kept at +5° C. for 1 to 1½ hours, after which 0.1 ml. of the mixture was added to each of 2 roller tubes. Controls of virus plus buffer solution instead of serum were included with each test and in many of the experiments known positive serum controls also were included. Pools of infective tissue culture fluid used for neutralization tests were stored in ampules in dry ice and a titration was carried out with each test. The acute phase and the convalescent serum from a given patient were tested simultaneously. Inoculated cultures were generally changed to fresh media only once during an observation period of 10 to 12 days. Further details regarding neutralization tests are included in the text.

EXPERIMENTAL

Isolation of Agents From Clinical Specimens.—

From the community outbreak: Stool specimens from 11 clinically diagnosed cases of Boston exanthem disease were tested in cultures of either monkey kidney or testicular tissues. Fecal specimens were collected within 2 to 10 days after onset of illness. Serially transferable agents, which produced cytopathogenic effects indistinguishable from one another, were isolated from all 11 patients. Each isolate was passed at least once in both kidney and testicular cultures with the production of characteristic types of cellular destruction.

Either throat or mouth washings, collected within 3 days of onset of illness, were also available from 3 of the 11 patients whose stools were tested. Similar agents were recovered from throat washings of 2 of the cases (Mr. McG. and Mr. W.), but mouth washings from the third case (R. W.) were negative. A fourth patient whose throat washings were tested (Mrs. W.), but from whom no stool specimen was available, was negative. This patient had a characteristic clinical illness and was the only case of those tested in the community outbreak in whom isolation of a cytopathogenic agent was not achieved.

Recovery of virus from a stool specimen of a family contact, who also was reported to have had a characteristic illness with rash, constituted the twelfth isolation of an agent in association with the suburban community outbreak.

Isolations from subsequent sporadic cases: Stool specimens from 6 cases which occurred later in 4 widely separated areas of the city were tested in cultures of monkey kidney tissue. In each instance an agent was recovered which produced cytopathogenic changes similar to those isolated from the community outbreak. Data summarizing the origin of virus isolations from all the Boston exanthem cases in Pittsburgh are presented in Table I.

Relative susceptibility of several different tissues for primary isolation: Most specimens were first tested in roller cultures of monkey kidney, but all negative and most positive specimens were also tested in monkey testicular cultures. It soon became apparent that testicular cultures were more sensitive indicators of the cytopathogenic effects of these particular agents than were the kidney cultures.

TABLE I. VIRUS ISOLATIONS AND SERUM NEUTRALIZATION TESTS IN PITTSBURGH PATIENTS WITH BOSTON EXANTHEM DISEASE

PATIENT	SEX	AGE IN YEARS	VIRUS ISOLATION		SERUM NEUTRALIZATION TESTS*		
			MATERIAL TESTED	RESULT	DAY OF DISEASE	TITER VS. PITTSBURGH STRAIN	TITER VS. BOSTON STRAIN
Cases from Community Outbreak							
S. McG.	F	5/12	Feces	+	No specimens obtained		
D. McG.	M	4	Feces	+	6	4	2
					27	16 to 32	16
R. McG.	M	6	Feces	+	6	4	4 to 8
					27	16	32
Mr. McG.	M	29	Blood clot	+	2	<2	<2
			Throat wash.	+	23	8	4
			Feces	+			
R. Wel.	M	5	Blood clot	0	3	<2	<2
			Mouth wash.	0	20	8 to 16	8
			Feces	+			
K. Wel.	M	6	Blood clot	0	3	<2	<2
			Feces	+	20	8	16
Mrs. Wel.	F	30	Blood clot	0	2	<2	<2
			Throat wash.	0	17	32	16
Mr. Wol.	M	30	Blood clot	0	2	–	2
			Throat wash.	+	23	–	16
			Feces	+			
Mrs. Wol.	F	29	Feces	+	4	2	4
					27	8	16
Mrs. B.	F	28	Blood clot	0	4	2	<2
			Feces	+	21	32	16
Mr. D.	M	35	Feces	+	3	<2	<2
					18	32	16
T. F.	M	5	Feces	+	5	2	<2
					26	8	4 to 8
L. L.	F	6	None	–	9	8	>8
					28	32	>32
J. P.	F	2	Feces	+	No specimens obtained		
Sporadic Cases							
A. J.	F	2/12	Feces	+	No specimens obtained		
Mrs. J.	F	36	Feces	+	5	4	4
					22	8	4
A. R.	F	7/12	Blood clot	0	3	2	<2
			Feces	+	20	4	8
E. N.	M	1	Blood clot	0	4	<2	<2
			Feces	+	18	4 to 8	8
E. W.	M	8/12	Blood clot	0	5	<2	2
			Feces	+		No second specimen	
B. T.	M	2	Blood clot	0	?	4	4
			Feces	+	?	16	16

*Done in tissue cultures, using 5 to 50 ($TCID_{50}$) of virus. Titers are reciprocal of serum dilution before virus was added. Pittsburgh strain was isolated from R. Wel. in 1954. Boston strain was isolated during 1951 outbreak in Boston, Mass.

Seven specimens, positive in kidney, were also positive in testicular cultures. However, 4 specimens negative in kidney were found positive when comparable inocula were tested in testicular cultures. In addition, several other specimens produced questionable effects on the first passage in monkey kidney and required a second passage for definite cytopathic changes. In either type of culture, first evidence of cytopathogenic changes was noted between the third to the eleventh day after inoculation.

The difference in susceptibility between monkey kidney and testis was also evident when some specimens were titrated for infectivity in both types of cultures. Ten per cent stool suspensions from several patients were found to contain detectable virus when diluted $10^{-3.0}$; in general, titers in monkey kidney were tenfold to a hundred fold lower than those in testicular cultures. Data on the comparative susceptibility of monkey kidney and testicular tissue for primary isolation are summarized in Table II.

TABLE II. SUSCEPTIBILITY OF MONKEY TESTICULAR VERSUS KIDNEY CULTURES TO AGENTS ISOLATED FROM EXANTHEM PATIENTS

PATIENT AND SPECIMEN	MONKEY KIDNEY CULTURES			MONKEY TESTIS CULTURES		
	INOCULUM		RESULT*	INOCULUM		RESULT
Mr. McG. Blood Clot	0.25 ml. to 5 tubes		0/5 Positive	0.4 ml. to 4 tubes		4/4 Positive
Mr. McG. Feces	0.1 ml. to 4 tubes		0/4 Positive	0.1 ml. to 3 tubes		2/3 Positive
Mr. McG. Throat wash.	0.4 ml. to 5 tubes		1/5 Positive	0.5 ml. to 4 tubes		4/4 Positive
K. Wel. Feces	0.1 ml. at	10^{0}	3/3 Positive	0.1 ml. at	10^{-1}	2/2 Positive
		10^{-1}	0/3 Positive		10^{-2}	3/3 Positive
		10^{-2}	0/3 Positive		10^{-3}	0/4 Positive
A. Roh. Feces	0.1 ml. at	10^{-1}	1/3 Positive	0.1 ml. at	10^{-1}	2/2 Positive
		10^{-2}	0/3 Positive		10^{-2}	3/3 Positive
		10^{-3}	0/3 Positive		10^{-3}	1/4 Positive

*Numerator represents the number of cultures showing degeneration; the denominator is the number of tubes inoculated.

Subsequent experiments[5] using only slight modifications in nutrient media* with cultures of different types of human tissue have revealed the following comparisons of susceptibility to these agents: several different strains tested in trypsinized cultures of human kidney produce minimal or no obvious cytopathogenic changes, but further passage in these tissues may increase cytopathic effects. Human foreskin and embryonic skin-muscle cultures are approximately equal in susceptibility, either upon primary isolation or after passage. Trypsinized human amnion cultures show variable degrees of cytopathic changes, but are less susceptible than human fibroblasts. Cultures of HeLa cells have shown no obvious changes up to 12 days after inoculation.

Attempts to isolate agents from the blood of patients: Frozen suspensions of clotted blood from 10 patients, bled on the second to fifth day of disease,

*The media consisted of 90 per cent bovine amniotic fluid, 5 per cent horse serum, and 5 per cent beef embryo extract.

were tested in roller cultures of monkey testis. Inocula per tube varied from 0.2 to 1.25 ml., and in many instances a further blind passage was carried out. An agent similar to those isolated from the feces was recovered from only one blood specimen (Mr. McG.). In this instance each of 4 tubes inoculated with 0.3 ml. produced characteristic changes within 5 to 7 days. It is of interest that the blood from the positive case was drawn when the patient had a fever of 104° F. while all negative bloods were from afebrile patients even though collected on the second or third day of illness.

Tests for Neutralizing Antibody.—

General comments regarding neutralization tests in tissue culture: It has been observed previously[6] that neutralization of the exanthem agents by human convalescent serum was difficult to demonstrate unless minimal amounts of virus were used in the neutralization mixture. Even when 50 $TCID_{50}$ of virus are employed in a neutralization test, the phenomenon of virus "breakthrough," or progressive decrease in titer of a serum is seen with the passage of time.

The possibility was considered that changing the media of cultures during the test might contribute to the incomplete neutralization observed above. However, identical tests in which one set of cultures was changed and one set was kept with the original medium showed no striking difference in the results. Conditions of the test were further modified in an attempt to obtain more complete neutralization of virus by specific antibody. Incubation of the serum-virus mixture for one hour at 37° C. instead of at refrigerator temperature failed to enhance neutralization. The use of graded dilutions of virus with a constant serum dilution likewise appeared to offer no particular advantage.

In view of the above enumerated limitations, the only available test at present for in vitro neutralization has utilized the criterion of 2 to 3 days' delay in appearance of cytopathic changes afforded by convalescent as compared to acute phase or control serum when tested at the same time. Under these conditions, values of antibody titer are necessarily arbitrary. However, upon the basis of numerous tests, presence or absence of neutralizing antibody can be readily determined and reproducible estimates of titer have been possible.

Neutralizing antibody response in patients: Paired samples of acute and convalescent phase sera were available from a total of 16 cases with clinical evidence of Boston exanthem disease. Twelve of these patients represented cases from the community outbreak, and 4 were later scattered cases. Acute and convalescent sera from each case were tested simultaneously for their ability to neutralize 2 different strains of virus. One of the strains had been isolated from feces of an exanthem patient in Pittsburgh during 1954, the other strain originated from feces of an exanthem patient in Boston during the 1951 outbreak. Five to fifty $TCID_{50}$ of virus were used in the tests. Serologic results, along with other information concerning the patients, are presented in Table I.

The neutralizing antibody response in patients to a Pittsburgh strain as compared with a Boston strain showed close agreement. In most cases a fourfold or greater rise in antibody titer could be demonstrated to both strains, and only one patient failed to show significant development of neutralizing antibody to at least one of the strains. In this case (Mrs. J.) antibody was already present

in the acute phase serum, but the onset of illness was so poorly defined that duration of disease could not be accurately determined.

*Neutralizing antibody response in monkeys**: The development of neutralizing antibody in either cynomolgus or rhesus monkeys could be demonstrated after primary intravenous or intracerebral inoculation followed by 2 intramuscular doses of infectious tissue culture fluid over a period of several months. This was shown with 6 different strains representing isolates from exanthem patients in Pittsburgh as well as cases from the first outbreak in Boston. Sera from 13 normal monkeys were negative for neutralizing antibody when tested at a 1:2 dilution. Neutralization of heterologous strains with a given monkey antiserum has been demonstrated, but the extent of antigenic relationship is still under investigation.

Failure to Associate Exanthem Agents With Other Illnesses.—During the year preceding the outbreak of exanthem disease cases in Pittsburgh, stools from approximately 90 patients with suspected poliomyelitis from that same city were studied. These were tested† in cultures of monkey kidney, and although a few unclassified cytopathogenic agents were isolated, none resembled the agents described herein. During the same season as the exanthem outbreak at least 30 fecal specimens from poliomyelitis suspects were likewise negative in monkey kidney culture for agents similar to those recovered from exanthem patients.

Negative results for either enteric cytopathogenic viruses or specific neutralizing antibody responses were obtained in the following illnesses: 3 patients with roseola infantum, 3 patients with German measles, and 6 patients with fever and unclassified skin rashes. Similar studies of 3 patients with vague minor illnesses in residents of the same community where cases of Boston exanthem disease occurred were also negative.

Serum Survey for Neutralizing Antibody to Boston Exanthem Agents.—

Results with gamma globulin: One lot of human immune serum globulin, and one lot of gamma globulin from convalescent German measles blood, both of recent origin, were tested.‡ Neither of the lots had detectable neutralizing antibody to one of the exanthem strains at dilutions greater than 1:10. Six other lots of outdated gamma globulin were also tested; only one of the lots showed a detectable level of neutralizing antibody at 1:10, the lowest dilution tested. However, these latter preparations may have decreased in potency with prolonged refrigerator storage.

Results with sera from different age groups: Serum samples collected in 1953 and 1954 from the Pittsburgh area from patients with poliomyelitis and other illnesses were tested for neutralizing antibody to a Boston strain of exanthem agent. Each serum was inactivated and tested at a 1:2 dilution with 50 $TCID_{50}$ of virus in 2 roller cultures.

The results are summarized in Table III. Since there was no striking difference in incidence of neutralizing antibodies from sera collected in 1953

*These experiments were done jointly with Dr. L. James Lewis.

†Many of these specimens were tested by Dr. Gisela Ruckle in this laboratory.

‡These lots of gamma globulin were kindly made available by Dr. Johannes Ipsen, Institute of Laboratories of the Massachusetts Department of Public Health.

as compared to the 1954 specimens, the number tested in each age group represent both years of collection. Neutralizing antibody could be demonstrated in 5 per cent of the group less than 5 years old, 19 per cent of those 5 to 15 years old and in only 4 per cent of the group over 15 years of age. Some of the sera showed equivocal neutralization. This partial neutralization was found in only one or two specimens from either of the younger age groups, but 7 (15 per cent) of those over 15 years of age gave a doubtful result. Repeat tests with a number of the sera showed no change from the original results.

TABLE III. PRESENCE OF NEUTRALIZING ANTIBODY TO BOSTON EXANTHEM AGENT IN PITTSBURGH SERA, 1953-1954

	AGE GROUP		
	< 5 YEARS	5-15 YEARS	> 15 YEARS
Number tested	39	62	45
Number positive	2 (5.1%)	12 (19.4%)	2 (4.4%)
Number doubtful	1 (2.6%)	2 (3.2%)	7 (15.5%)

DISCUSSION

The viruses described were readily isolated from the feces of almost every Pittsburgh patient with the clinical picture of Boston exanthem disease. Throat washings, in contrast, appeared to be less suitable for recovery of these agents. Isolation from the blood was successful in only one of many attempts suggesting either that viremia occurs rarely, or that the blood specimens tested were obtained too late in the course of the illness.

The differences of in vitro sensitivity among various tissues must be kept in mind if isolation of these agents is attempted. Cultures of monkey kidney were adequate in most instances for primary isolation, although they were less susceptible than monkey testis. Human kidney cultures showed either no, or only equivocal, cytopathic effects. Cultures of human embryonic skin muscle or foreskin have shown approximately the same susceptibility as monkey testis.

Tests for neutralizing antibody in sera from Pittsburgh exanthem patients showed essentially the same results when tested with a Pittsburgh strain of virus as with a strain representing the earlier Boston outbreak. Thus, close antigenic relationship was demonstrated between agents recovered from patients with similar diseases that occurred several years apart in two widely separated cities.

The neutralization test with this group of viruses is still not entirely satisfactory. With the methods described, it is possible to obtain fairly reproducible evidence of neutralization. However, the factor of incomplete neutralization, or virus "breakthrough," necessitates arbitrary interpretation of the test, and constitutes a drawback in serologic work.

The results of the screening tests for neutralizing antibodies suggest that a moderate incidence of exposure to these agents had occurred during childhood. The low incidence of definitely positive sera and the relatively large number of doubtful results in the adult age group is more difficult to interpret. However, this may indicate a decline in levels of neutralizing antibody from infections acquired during childhood, as well as a low incidence of infection in

adults. The results of tests for antibody in gamma globulin are consistent with the low incidence of definitely positive sera encountered in the screening of adults, and also agrees with the relatively low antibody titers found even in recently convalescent patients.

A high degree of association has been demonstrated between a recognizable clinical entity and the specific agents recoverable from such patients. This relationship of agents to disease was found not only in the setting of a localized outbreak, but also in scattered sporadic cases occurring in a large metropolitan area. The agents isolated from patients with the Boston exanthem disease could not be recovered from certain cases of other known or unclassified febrile exanthems, or from many patients with other illnesses which occurred in the same area at approximately the same time. A similar association of related or identical agents with the same type of illness has been reported previously.[6] These observations strongly suggest an etiologic relationship between this group of viruses and the illness referred to as Boston exanthem disease.

Further information, however, is needed to establish conclusively the etiologic role of these agents. The number of patients who have been studied may be insufficient to fully define the range of possible clinical manifestations. It is not yet known whether infection with these agents is invariably associated with a specific clinical illness. Finally, the existence of other related agents, as yet unknown, cannot be excluded. For example, it has been found recently[7] that certain enteric viruses recovered from several patients with aseptic meningitis that occurred during the first reported epidemic of Boston exanthem disease are related immunologically to the viruses described in this paper. The extent of immunologic relationship and comparison of other characteristics is under investigation.

SUMMARY

Study of a 1954 outbreak of Boston exanthem disease in Pittsburgh resulted in the isolation of characteristic agents cytopathic for tissue cultures from 18 patients. The viruses were recovered most readily from the feces, irregularly from throat washings, and once from the blood of patients. Cultures of monkey testicular tissue were found to be superior to those of monkey kidney for primary isolation. Neutralizing antibody responses in Pittsburgh patients indicated a close antigenic relationship of local strains to similar agents associated previously with the same type of disease in Boston. Results of screening tests of various age groups for neutralizing antibody are presented. The observations presented strengthen the likelihood that these viruses are etiologically related to the Boston exanthem disease.

We are indebted for advice to Dr. Jonas E. Salk in whose laboratory these studies were carried out.

REFERENCES

1. Neva, F. A., Feemster, R. F., and Gorbach, I. J.: J. A. M. A. **155:** 544, 1954.
2. Neva, F. A.: New England J. Med. **254:** 838, 1956.
3. Salk, J. E., Youngner, J. S., and Ward, E. N.: Am. J. Hyg. **60:** 214, 1954.
4. Youngner, J. S.: Proc. Soc. Exper. Biol. & Med. **85:** 202, 1954.
5. Neva, F. A., and Malone, M. F.: Unpublished observations.
6. Neva, F. A., and Enders, J. F.: J. Immunol. **72:** 307, 1954.
7. Kibrick, S.: Ann. New York Acad. Sc. **67:** 311, 1957.

LABORATORY METHODS

THE COLLECTION OF LYMPH FROM INTACT PERIPHERAL CUTANEOUS LYMPHATICS OF MAN

SAM A. THREEFOOT, M.D.
NEW ORLEANS, LA.

AS PART of a study of the role of the lymphatics in the formation of edema in congestive heart failure, a method was developed for the cannulation and collection of fluid from intact cutaneous lymphatics of the extremities of man. Whereas the chemical content of lymph from animals and from the thoracic duct of man has been studied extensively,[1, 2] there are relatively few data concerned with the electrolyte content and lymphodynamics of intact lymphatics in the extremities of man. The method presented is applicable to these as well as other studies of peripheral lymphatics and lymph under various circumstances without severe disturbance of the locale studied.

MATERIALS AND METHODS

The lymph channels are first visualized by the intradermal injection of 0.1 ml. sterile 11 per cent aqueous solution of Patent Blue V* with a 30 gage needle.[3-5] This quantity produces streamers intense enough for cannulation in 10 to 20 minutes. The longest and most intense streamers are most commonly seen on the forearm of normal subjects, whereas in edematous extremities there is rapid diffusion of the dye and distinct channels are sometimes difficult to discern.[5] The use of red fluoroscopic glasses facilitates the visualization of the blue-stained lymphatics and differentiation from veins. All studies were performed after 30 minutes horizontal rest of the extremity at 76° F. and no attempts were made to induce or hasten streamer formation by exercise or application of heat to the part.[4]

Cannulation is performed counter to the current of flow with either a 23 gage needle from which the hub has been removed or a one-inch 30 gage needle modified by removing the hub and soldering it into a ½ inch length of 23 gage stainless steel tubing (Figs. 1, 2, and 3). A suitable length of plastic tubing designed to fit snugly on a 23 gage needle† is attached as a collecting vessel with a U-tube phlebomanometer[6] as an aspirator and indicator of the negative pressure applied. A three-way stopcock permits recompression of the bulb, if necessary, without raising the pressure in the collecting system. If a syringe is used for aspiration, care must be taken not to exert a pressure so great as to collapse the small vessel or draw air into the plastic tubing through the needle puncture site or any of the junctions in the system. When this occurs a broken column of fluid is obtained, handicapping volume estimation, permitting evaporation from a greater surface area, and

From the Department of Medicine, Tulane University School of Medicine and Research Laboratory, Touro Infirmary, New Orleans, La.

Supported by research grant H-1520 from the National Heart Institute of the National Institutes of Health, Public Health Service, by the Louisiana Heart Association, The John A. Hartford Foundation, and the Jacob Aron Research Fund.

Received for publication March 29, 1957.

*National Aniline Division, Allied Chemical & Dye Corporation.

†B-D 442T.

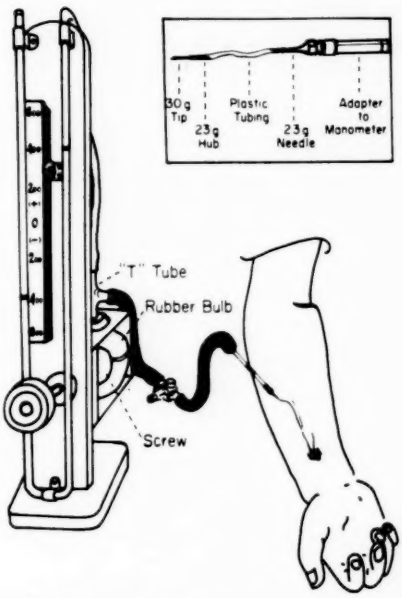

Fig. 1.—The technique of cannulating a cutaneous lymphatic and collecting lymph. The phlebomanometer serves as a regulated aspirator. The 3-way stopcock in the line permits recompression of the bulb if the pressure falls due to leakage in the system. The insert shows the modified 30 gage needle attached to the collecting tubing and adaptor to aspirator. A 23 gage needle with the hub removed can be used as the cannula just as well.

Fig. 2.

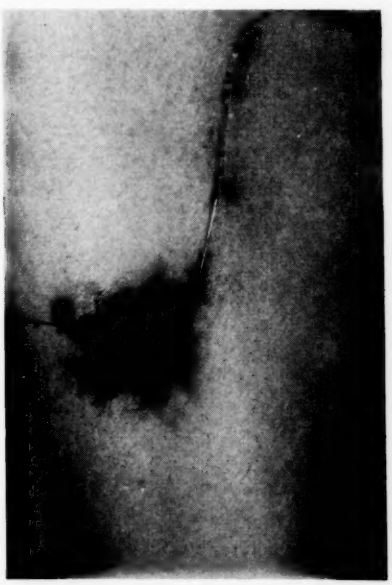

Fig. 3.

Fig. 2.—Collection of lymph from a streamer in the arm of Subject 23D (CHF). This picture was taken 70 minutes after injection of the dye and 19 minutes after collection of lymph was begun. Stained lymph can be seen in the plastic tubing just above the cannula.

Fig. 3.—Collection of lymph from the leg of Subject 24 (CHF) illustrating undesirable fragmentation of the column of fluid by air pulled into the collecting tubing during forced aspiration.

increasing the difficulty of expelling the fluid from the plastic tubing (Fig. 3). This is rarely encountered when a negative pressure of 200 to 500 mm. of water is applied, but the time required to determine whether or not the vessel has been entered is longer. Since the rates of flow are small, 5 to 10 minutes should elapse before the investigator assumes he is not in the lymphatic and spoils a good cannulation by premature withdrawal of the needle.

If an unbroken column of fluid is collected in the plastic tubing, the quantity can be estimated periodically during the collection by measuring the length of the column. Measurements under controlled conditions showed the relationships of the weight of the fluid delivered to the length of a fluid column in the tubing to be 0.25161 mg. per millimeter ± 0.00215 (Fig. 4). For precise measurement of the quantity of fluid delivered for chemical determinations, the tubing is weighed before and after the fluid is expelled into the desired container. Loss of fluid by evaporation from the open ends of the tubing during the interval before weighing may be reduced by drawing the fluid a short distance from the distal end and clamping. The rate of evaporation from tubing so clamped was measured at 76° F. and found to average 0.6 per cent per hour for a 24-hour period.

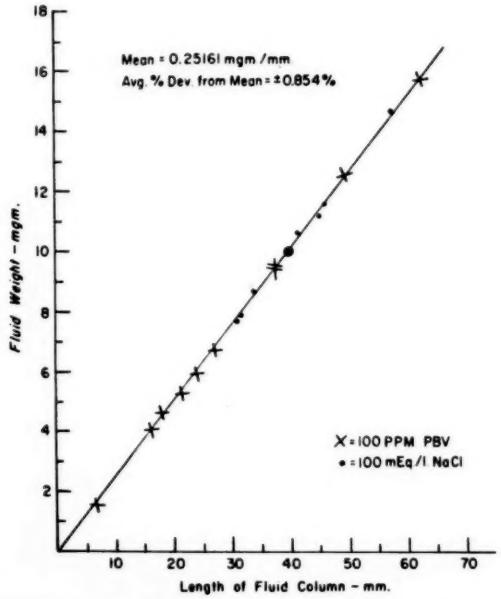

Fig. 4.—Relationship of the weight of fluid delivered from the plastic tubing to the length of the column of fluid in the tubing (B-D 442 T). Each millimeter of fluid in the tubing weighs 0.25161 mg. There were no detectable differences when PBV, sodium chloride, or serum was used for calibration.

In the determination of the electrolyte content of the lymph, contamination of the small volume collected has been minimized by expelling the lymph from the plastic tubing into siliconed 4 ml. weighing bottles which have been rinsed at least 10 times with triple distilled water and dried in room air while covered to protect from dust particles. Triple distilled water is also used as the diluent. Concentration of sodium in such samples has been determined with a Beckman flame photometer and concentration of chloride by potentiometric titration with silver nitrate of a 1 ml. aliquot of that diluted for sodium determination, evaporated to dryness and redissolved in 0.2 ml. 50 per cent acetic acid.[7, 8]

The concentration of radioactive tracers has been determined by delivered onto disks for counting.[9] Pressures within lymphatics have been measured by the Burch technique for venous and tissue pressures.[10]

RESULTS

The number of experiments, subjects studied, and cannulations is presented in Table I. Lymphatics were entered in 62.6 per cent of the attempted cannulations as evidenced by the appearance of the blue-tinged lymph in the tubing or at the site of the puncture after withdrawing the needle. In 36 instances enough lymph was obtained for gravimetric quantification. In 15 (13 per cent) of the cannulations blood was obtained from vessels which contained dye and which were indistinguishable by inspection from streamers which yielded lymph.

TABLE I. TABULATION OF COLLECTION OF LYMPH

	CONTROLS		CHF		OBSTRUCTIVE		MISC.		TOTALS		
	ARM	LEG	ARM	LEG	ARM	LEG	ARM	LEG	ARM	LEG	TOTAL
Number subjects	11	4	3*	5*	0	3	1†	3‡	15*	15*	28*
Number experiments	13	4	3	5	0	4	1	3	17	16	33
Attempted lymphatic cannulations	43	20	9	21	0	11	2	9	54	61	115
Lymphatics entered	27	9	6	11	0	9	2	8	35	37	72
Quantity sufficient for measurement	8	5	5	7	0	5	1	5	14	22	36
Blood obtained from streamer	3	3	2	5	0	1	0	1	5	10	15

CHF = Congestive heart failure.
*Two subjects were studied in the leg on one occasion and in the arm on repeated study.
†Chronic glomerulonephritis, no edema.
‡One edema, undiagnosed etiology; 1 cirrhosis, edema; 1 myocardial infarction, no failure, no edema.

The rates of collection of lymph, according to the type of subject and size of the cannula, are presented in Fig. 5. This figure and the time courses of the cumulative collection of lymph from some of the subjects shown in Fig. 6 illustrate that the rates of collection were greatest and volumes collected largest from the edematous legs of subjects with congestive heart failure when a 23 gage needle was used. The quantities of lymph collected from all subjects varied with the duration and rate of collection and ranged from 0.15 mg. to 19.0 mg.

Twenty-nine samples collected were analyzed for sodium. Values for the 18 samples analyzed after problems of obvious contamination were alleviated are presented in Table II, A. The concentrations of chloride determined by the micropotentiometric titration are included in this table. In 9 studies edema fluid was obtained directly without cannulating the lymphatics. The volumes collected in these instances were of sufficient magnitude to permit standard methods of determination of electrolyte or protein content (Table II, B).

The concentration of dye in the lymph of 5 subjects ranged from 0.005 to 6.7 per cent of the concentration injected. In three studies after radioiodinated serum albumin* was injected at the same site as the dye, concentration of I^{131}

*Risa, Abbott Laboratories, North Chicago, Ill.

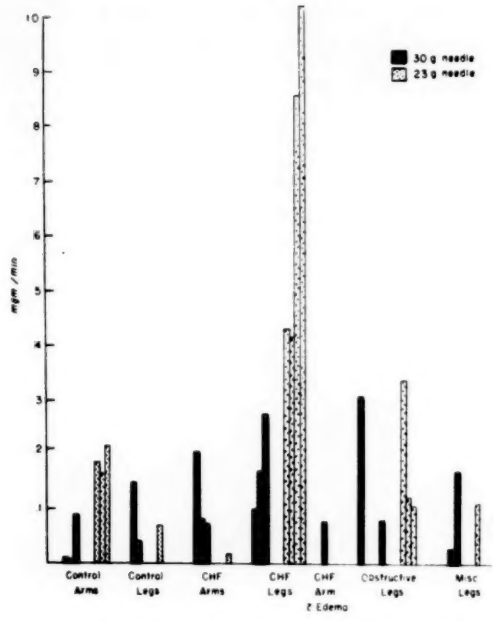

Fig. 5.—The rates of collection of lymph from various subjects with either a 23 or 30 gage needle. The greatest rates of collection were apparent when a 23 gage needle was used to collect from subjects with congestive heart failure.

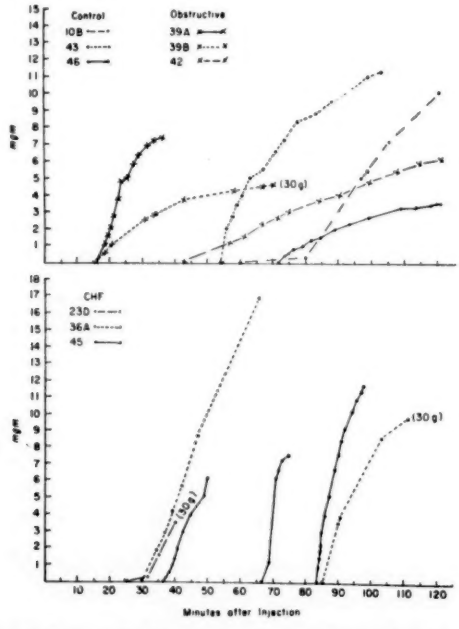

Fig. 6.—The cumulative collection of lymph demonstrating the time course of collection. A 23 gage needle was used except when noted on this graph. The collection appears to be fastest in subjects with congestive heart failure, as indicated by the steeper slopes of the lower set of curves. The weight in milligrams has been calculated from the column length using the calibration factor 0.25161 mg. per millimeter.

in the lymph varied from 0.005 per cent to 2.9 per cent of the concentration of the injected counts. Such variations resulted from differences in times of collection after injection and in distances from the injection site. These factors must be controlled before such data can be used to evaluate physiologic influences, such as variations produced by differences in uptake by the lymphatics or variations in rates of flow within the vessels. In one subject the concentration of dye in blood obtained from what appeared to be lymphatics was 2 per cent of the injected concentration of dye.

TABLE II, A. ELECTROLYTE CONTENT OF LYMPH

SUBJECT NO.	DIAGNOSIS	SITE	EDEMA	SODIUM (MEQ./1)		CHLORIDE (MEQ./1)		PROTEIN (GM. %)	
				SERUM	LYMPH	SERUM	LYMPH	SERUM	EDEMA
32	Control	Arm	0	143.5	148.2				
33	Control	Arm	0	140.5	148.2				
43	Control	Arm	0	140.0	138.8	106.0	107.3		
46	Control	Leg	0	143.5	154.5				
48	Control	Arm	0	140.5	138.8				
11-3	Control	Leg	0	141.7	138.6				
23D	CHF	Arm	4+	131.0	127.9				
			in leg	131.0	132.9				
36	CHF	Leg	3+	141.2	137.5				
				141.2	145.2	111.0	100.1		
				141.2	142.4				
45	CHF	Leg	3+	133.0	128.1				
39A (6/18)	Obstructive	Leg	0	139.5	150.3				
				139.5	135.2				
39B (6/25)	Obstructive	Leg	3+	136.7	137.3				
42	Obstructive	Leg	4+	143.2	153.7				
44	Cirrhosis	Leg	3+	126.5	127.6	95.0	101.9		
				126.5	129.2				

TABLE II, B. ELECTROLYTE CONTENT OF EDEMA FLUID

23A (10/4)	CHF	Leg	4+	134.0	137.8	97.5	102.5		
				134.0	142.5	97.5	105.6		
23B (10/18)	CHF	Leg	4+	125.0	129.0	100.6	109.7		
23C (11/14)	CHF	Leg	4+	129.2	131.2	88.3	95.9		
23D (12/13)	CHF	Leg	4+	131.0	131.4				
				131.0	131.0	91.1	99.6	5.75	0.54
19B	Obstructive	Arm	4+	144.0	124.5	102.8	107.6		*
11-1A (1/29)	Renal cancer	Leg	4+	131.3	131.3	86.2	95.2		
11-1B (1/31)	Renal cancer	Leg	4+	133.9	133.0	90.0	95.8	3.82	0.708
								A 2.35	A 0.576
								G 1.47	G 0.132

*Quantity insufficient for demonstration, but was thick, viscous, and yellow fluid.

On two occasions pressure within the lymphatics was determined with a phlebomanometer.[10] Because of slow flow and the small needles used, lags in the determination permitted only an estimate of the pressure within limits of 20 to 30 mm. of water in a normal subject and 50 to 75 mm. of water in a subject with obstructive lymphedema and venous disease. More accurate methods of determination are being adapted for use.

DISCUSSION

Although Patent Blue V was used for the visualization of the lymphatics in these studies, other dyes can be employed. Direct Sky Blue,* which has

*Wyeth, Inc., Philadelphia, Pa.

also been utilized satisfactorily in this laboratory, or other methods of visualization of the lymphatics, such as the use of fluorescent substances and ultraviolet light,[11] may be preferred by individual investigators. Although most of the blue streamers which developed after the injection of Patent Blue V were lymphatics, some proved to be blood vessels as demonstrated by the collection of blood containing dye and by serial photographs showing intensification of the color of small veins as dye entered them.[12] It is unlikely that this was due to the direct injection of venous channels, since from 5 to over 20 minutes were required for these vessels to become intensely blue or to develop the appearance of lymphatic streamers. These observations indicate that veins as well as lymphatics have been included in studies demonstrating "lymph channels" and measuring the rates of spread of dye.[3-5, 11] These studies also confirm the suggestion of Kinmonth that Patent Blue V is taken up by venous capillaries.[13]

The quantity of data thus far collected by this technique is not sufficient for interpretative evaluation of the variations observed. There are some observations, however, deserving comment. In Table II, A it may be noted that some of the values for the concentration of sodium in lymph were higher than those in serum. Although a "control" serum was run with each sample of lymph there was no assurance against minute undetectable contamination of individual beakers used for the dilutions, which would yield results higher than the true concentration. Collection of more than one sample from the same individual as control of this was possible in some subjects (Table II, A). This type of error did not exist in the determination of the concentration of sodium in the serum or edema fluid since relatively large volumes of sample were available.

The rates of collection of lymph illustrated in Figs. 5 and 6 cannot be interpreted as indicating rates of lymph flow within the lymphatics since these studies were not designed to control all of the factors known to influence these rates of collection. The slightly faster rates of collection from subjects with edema, however, suggest that the size of the "lymph pool" or elevation of tissue pressure and pressure within the lymphatics plays a part in the facilitation of collection of lymph from edematous extremities regardless of flow within the vessels themselves.[5] Further investigation is designed to study the influence of these factors upon the rate of collection and to study rates of flow within the lymph vessels.

SUMMARY

A method for the cannulation and collection of fluid from the cutaneous lymphatics of the extremities of man after visualization of the lymphatics by the intradermal injection of a vital dye has been presented. In 33 experiments 115 lymphatic cannulations have been attempted. The lymphatics were entered 72 times, in one-half of which the quantity of lymph collected was sufficient for measurement. Blood was obtained on 15 occasions from streamers which appeared to be lymphatics, indicating that some studies of visualized lymphatics have probably included small venous channels as well. Since only a small

amount of data concerned with the electrolyte content of lymph has been collected, no attempt has been made to evaluate differences between subjects. It has been pointed out that due to the small volumes of fluid and hazards of contamination the possibility of errors in these determinations exists. Concentrations of electrolytes determined by standard techniques on larger volumes of edema fluid are presented for comparison.

When a small volume of fluid is not a limiting factor, the collection of lymph by this technique has potential usefulness in the study of cytologic content, transport of crystalloids, colloids or tracer substances, antibody responses, or other processes in conditions not necessarily related to edema.

The technical assistance of Miss Patricia J. Grubbs and Mrs. Mary J. Smith is acknowledged.

REFERENCES

1. Yoffey, J. M., and Courtice, F. C.: Lymphatics, Lymph, and Lymphoid Tissue, Cambridge 1956, Harvard University Press.
2. Bierman, H. R., Byron, R. L., Kelly, K. H., Gilfillan, R. S., White, L. P., Freeman, N. E., and Petrakis, N. L.: The Characteristics of Thoracic Duct Lymph in Man, J. Clin. Invest. **32**: 637-649, 1953.
3. Hudack, S. S., and McMaster, P. D.: The Lymphatic Participation in Human Cutaneous Phenomena, J. Exper. Med. **57**: 751-774, 1933.
4. McMaster, P. D.: Changes in the Cutaneous Lymphatics of Human Beings and in the Lymph Flow Under Normal and Pathological Conditions, J. Exper. Med. **65**: 347-372, 1937.
5. McMaster, P. D.: The Lymphatics and Lymph Flow in the Edematous Skin of Human Beings With Cardiac and Renal Disease, J. Exper. Med. **65**: 373-392, 1937.
6. Burch, G. E., and Sodeman, W. A.: The Estimation of the Subcutaneous Tissue Pressure by a Direct Method, J. Clin. Invest. **16**: 845-850, 1937.
7. Sanderson, P. H.: Potentiometric Determination of Chloride in Biological Fluids, Biochem. J. **52**: 502-505, 1952.
8. Hyman, E. S.: Personal Communication, 1956.
9. Burch, G. E., Reaser, P., Ray, C. T., and Threefoot, S. A.: A Method of Preparing Biologic Fluids for Counting of Radioelements, J. Lab. & Clin. Med. **35**: 626-630, 1950.
10. Burch, G.: A Primer of Venous Pressure, Philadelphia, 1950, Lea & Febiger, p. 92.
11. Gifford, R. W., Jr., Windesheim, J. H., Estes, J. E. Jr., and Roth, G. M.: Fluorescent Patterns of Intracutaneous Wheals in Normal and Edematous Extremities, Circulation **13**: 515-523, 1956.
12. Threefoot, S. A.: Unpublished data.
13. Kinmonth, J. B.: Lymphangiography in Man—A Method of Outlining Lymphatic Trunks at Operation, Clin. Sc. **2**: 13-20, 1952.

THE DETERMINATION OF SERUM ALBUMIN AND GLOBULIN BY A NEW METHOD

J. R. Debro, A.B., H. Tarver, Ph.D., and A. Korner, Ph.D.*
Berkeley, Calif.

INTRODUCTION

RECENTLY two of us[1] showed that serum albumin, after precipitation with trichloroacetic acid (TCA), is soluble in alcohol. Moreover, albumin with similar physical properties to the native material can be recovered from the alcoholic TCA solution. The globulins, on the other hand, are completely denatured and are quite insoluble in alcoholic TCA. Later it came to our attention that the same type of observations had been made previously by Delaville and co-workers[2, 3] and Levine.[4] More recently this phenomenon has been investigated extensively by Schwert[5] who showed that the product obtained by solubilization in alcoholic TCA retains the same characteristics as albumin with respect to electrophoresis, sedimentation, crystallization, solubility, and thermal stability. We have made similar observations with respect to the first two of these characteristics, some of which we wish to report in this communication.

These observations make it possible to design a very simple method for the estimation of serum albumin and globulin, a method which can be readily adapted to a micro scale without loss of accuracy. This is made possible by applying the elegant method of Lowry and co-workers[6] to the measurement of the protein and the albumin solubility in TCA to separate serum albumin from the globulins.

MATERIALS AND METHODS

Reagents.—

Trichloroacetic acid: 15 per cent (micromethod).

Trichloroacetic acid: 1 per cent in 96 per cent alcohol (macromethod).

Sodium carbonate: 2 per cent in 0.1 N NaOH.

Copper sulfate: 0.5 per cent $CuSO_4.5H_2O$ in 1 per cent potassium (or sodium) tartrate.

Alkaline copper reagent: Mix 50 ml. of sodium carbonate reagent and 1 ml. of copper reagent. Make fresh each day.

Phenol reagent: (Folin-Ciocalteau phenol reagent.[7]) Place 100 Gm. sodium tungstate dihydrate, 25 Gm. sodium molybdate dihydrate, 700 ml. water, 50 ml. 85 per cent phosphoric acid, and 100 ml. conc. HCl in a 2 liter flask with a glass joint and equipped with a reflux condenser. Reflux gently for 10 hours, then add 100 Gm. lithium sulfate, 50 ml. H_2O, and a few drops of bromine. Boil for 15 minutes under the hood to remove the excess halogen, cool, and filter as necessary. Titrate a portion of the reagent with standard base using phenolphthalein indicator and, on the basis of the titer found, dilute the total volume so as to obtain a reagent 1 N in acid. The reagent should have a clear yellow color. Store in a glass stoppered bottle under dust-free conditions.

From the Department of Physiological Chemistry, University of California School of Medicine, Berkeley, Calif.

This work was supported in part by a grant from the U. S. Public Health Service.

Received for publication April 1, 1957.

*Present address: Department of Biochemistry, University of Cambridge, Cambridge, England.

Albumin standard: 1 per cent crystalline bovine serum albumin* in water.

Ethanol: 96 per cent.

Procedure.—

Macromethod. Total protein: 1. Measure 0.5 ml. of serum into a 10 ml. volumetric flask and dilute to volume. 2. Determine the chromogenic value on 10 lambda samples as described below.

Macromethod. Albumin: 1. Dilute 0.5 ml. of serum to 10 ml. with the alcoholic TCA solution. Mix well by inversion. 2. Centrifuge down the globulins. 3. Determine the chromogenic value on 10 lambda samples of the supernatant.

Micromethod. Total protein: 1. Wash 10 lambda of serum with a micro pipette into approximately 100 lambda of water contained in a 10 ml. volumetric flask. Dilute to volume. 2. Determine the chromogenic value on 1 ml. aliquots of the diluted serum.

Micromethod. Albumin: 1. Wash 10 lambda of serum into 100 lambda of water. 2. Precipitate with 200 lambda 15 per cent TCA. 3. Make to 1 ml. with alcohol (96 per cent). Shake well. 4. Centrifuge down the globulins. 5. Determine the chromogenic value on 100 lambda samples.

Development of color: 1. Make the aliquot of the protein containing solution up to 2 ml. 2. Add 5 ml. of the carbonate-copper reagent. 3. Mix and allow to stand 10 minutes. 4. Add 0.5 ml. of the diluted Folin-Ciocalteu reagent rapidly and mix well. 5. Make a reagent blank as above but omit the protein. 6. After 30 minutes read the optical density at 780 mμ in a spectrophotometer (or in the Klett with the red filter). 7. Subtract the value of the reagent blank from the unknown. 8. Compare with the standard albumin or use a standard curve to determine the protein.

TABLE I. EFFECT OF TCA AND ALCOHOL ON PROTEIN IN SUPERNATANT*

TCA NORMALITY	ALCOHOL (PER CENT) 70	90
	OPTICAL DENSITY	
0.312	0.094	0.096
0.390	0.0845	0.083
0.468	0.072	0.071
0.546	0.072	0.072
0.624	0.072	0.072
0.702	0.070	0.071
0.780	0.0715	0.072
1.0	0.071	0.071

*To 100 lambda of serum diluted 1 to 10 was added 200 lambda of TCA of the strength indicated in the first column. The volume was then made up to 1 ml. Color was generated as described in the text.

TABLE II. RECOVERY OF ALBUMIN ADDED TO SERUM

NO.	MIXTURE ALBUMIN* (ML.)	MIXTURE SERUM (ML.)	A/G RATIO CALCULATED	A/G RATIO FOUND
1	0.0	0.5		1.00
2	0.25	0.5	1.56	1.55
3	0.50	0.5	2.11	2.05
4	0.75	0.5	2.66	2.62
5	1.00	0.5	3.22	3.30
6	0.0	0.5		1.01

*Albumin 4.0 per cent.

RESULTS

When the amount of TCA or alcohol used in the method is varied over quite a wide range, there is little effect on the amount of soluble protein. This is shown by the data given in Table I. Rather similar results were reported by French workers.[2, 3]

*Pentex, Kankakee, Ill.

It has also been observed that there is little precipitation of protein when the albumin is allowed to stand in the alcoholic TCA solution at room temperature. The protein is precipitated, however, if the TCA is neutralized.

When a sera was mixed with different amounts of a standard albumin solution and the ratio of albumin to globulin was determined for each mixture, the results shown in Table II were obtained. It is seen that the calculated and observed A/G ratios are in all cases very close and quite within the experimental error of the method.

The albumin and globulin in a series of pathologic and normal sera were determined both by free electrophoresis in the usual barbiturate buffer at pH 8.6 and of ionic strength 0.1 at 4° C. in the Aminco-Stern apparatus,* and by the micro-TCA method. From the results, given in Table III, it may be seen that generally the two sets of results are in good agreement, only one sample serum (No. 5) giving values which differ significantly.

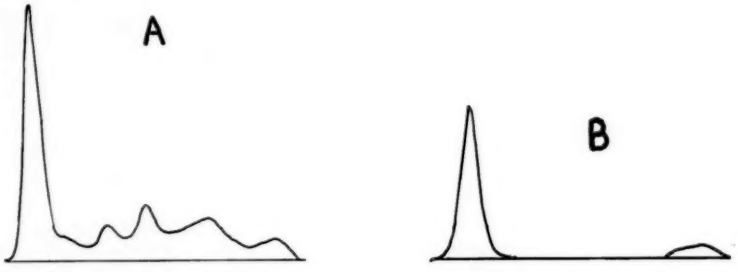

Fig. 1.—*A*, Electrophoretic patterns of whole serum. *B*, Albumin isolated by solubilization in alcoholic trichloroacetic acid.

TABLE III. COMPARISON OF ELECTROPHORETIC AND MICRO-TCA METHODS ON VARIOUS SERA

SUBJECT	PER CENT PROTEIN TOTAL	MICRO-TCA		ELECTROPHORESIS	
		ALBUMIN	GLOBULIN	ALBUMIN	GLOBULIN
1	9.13	4.62	4.51	4.57	4.56
2	8.56	4.86	3.70	4.67	3.89
3	6.76	4.15	2.61	4.16	2.60
4	8.90	4.62	4.28	4.62	4.28
5	8.00	2.85	5.15	2.40	5.60
6	6.20	2.90	3.30	3.04	3.16
7	8.00	2.52	5.48	2.33	5.67
8	7.57	2.85	4.72	2.75	4.82
9	6.00	3.10	2.90	3.27	2.73

We have separated the albumin from larger samples of several normal sera by the method indicated, obtaining a solution in alcoholic TCA. The albumin was recovered from this solution by dialyzing out the alcohol and TCA. In this process a small amount of insoluble protein separated. After removal of this material and subjecting the albumin to electrophoresis, only protein moving like albumin was found. This is shown in Fig. 1 which shows the diagram obtained with the original serum, and with the protein soluble in alcoholic TCA, and where the small peak is due to the false boundry. Thus the only

*We are obliged to Professor F. W. Allen for making this apparatus available to us and to Dr. A. M. Crestfield for instructions regarding its operation.

protein present in a significant amount in the serum, which is soluble in alcoholic TCA, appears to be albumin. At pH 8.5 the mobility is the same as that of pure crystalline albumin. However, at pH 5.4 the mobilities appear to diverge somewhat. For the crystalline material ascending and descending boundaries give mobilities of 3.05 and 3.33 $\times$ 10^{-5}, respectively, whereas the alcoholic TCA-treated material gives values of 2.7 and 2.99 $\times$ 10^{-5}.

TABLE IV. CHROMOGENIC VALUES OF SERUM PROTEIN

Whole serum (human)		2,060
Mercapt albumin (human)		2,130
Albumin (bovine)		2,160
Globulin (human):	alpha	2,130
	beta	2,030
	gamma	2,110

All values expressed in terms of equivalent extinction coefficient at 780 mμ, the optical density per atom N per liter.

Source of proteins: mercaptalbumin, Pentex; bovine albumin, The Armour Laboratories; Human globulins, Cutter Laboratories.

We are obliged to J. H. Hink Jr. of Cutter Laboratories for generously supplying us with samples of the various globulin fractions from human sera.

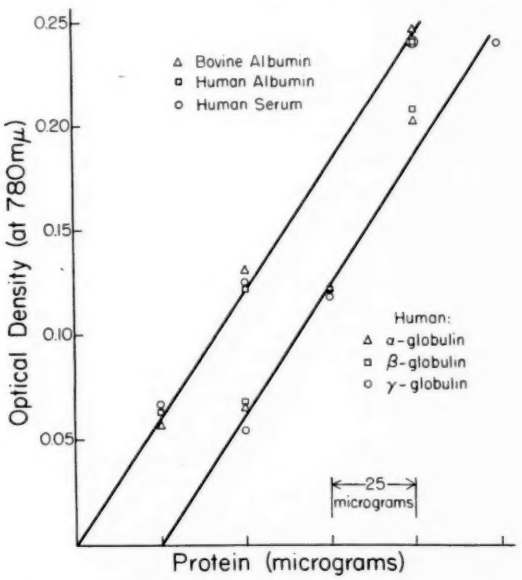

Fig. 2.—Optical density of solutions of various serum proteins after generating color by the method of Lowry and co-workers.

The chromogenic values of the different serum proteins have been examined and are recorded in Table IV and Fig. 2. The value of the extinction coefficient of bovine serum albumin and serum per atom nitrogen per liter at 750 mμ is given by Lowry and co-workers[6] as 2.05 and 2.12 $\times$ 10^{-3} as compared with the present values at 780 mμ of 2.16 and 2.06 $\times$ 10^{-3}. It is quite clear from these results that there is little difference between the present results and those of Lowry and co-workers[6] and between the several serum proteins. The close similarity in behavior of the proteins studied is shown more clearly still in Fig. 2.

DISCUSSION

It is quite evident that the method described for the determination of serum albumin is considerably simpler than any of those used at the present time in clinical work, and, since it can readily be carried out on a micro scale, it possesses considerable merit. Moreover, the method requires a minimum of reagents, only one of which is at all difficult to prepare and that one is available commercially (Folin-Ciocalteu reagent).

The color reaction used in this method is considerably more sensitive than the biuret reaction, and equivalent amounts to color are developed by all the serum proteins examined. A linear relationship exists between color developed and protein concentrations over a range of at least 25 to 125 μg of protein.

SUMMARY

A new micro method is described for the determination of serum albumin and globulin which depends on the solubility of the albumin in alcoholic trichloroacetic acid. The reagent renders the globulins insoluble by denaturation. Total protein and albumin in solution are determined by the use of the colorimetric method of Lowry and co-workers.

REFERENCES

1. Korner, A., and Debro, J. R.: Solubility of Albumin in Alcohol After Precipitation by Trichloroacetic Acid: A Simplified Procedure for Separation of Albumin, Nature **178:** 1067, 1956.
2. Delaville, M., Delaville, G., and Delaville, J.: J. Caractère de solubilité de la fraction albuminique du sérum sanguin dans l'alcool trichloracétique; son application au dosage des diverses fractions proteiques, Ann. pharm. franc. **12:** 109-113, 1954.
3. Delaville, M., Delaville, G., and Delaville, J.: Caractère de solubilité de la fraction albuminique du sérum sanguin dans les solutions D'ethanol trichloracétique; application au dosage des diverses fractions proteiques du sérum, Ann. biol. clinique **12:** 320-323, 1954.
4. Levine, S.: Solubilization of Bovine Albumin in Nonaqueous Media, Arch. Biochem **50:** 515, 1954.
5. Schwert, G. W.: Recovery of Native Bovine Serum Albumin After Precipitation With Trichloroacetic Acid and Solution in Organic Solvents, J. Am. Chem. Soc. **79:** 139, 1957.
6. Lowry, O. H., Rosebrough, N. J., Farr, A. C., and Randall, R. J.: Protein Measurement With the Folin Phenol Reagent, J. Biol. Chem. **193:** 265-275, 1951.
7. Folin, O., and Ciocalteu, J.: On Tyrosine and Tryptophane Determinations in Proteins, J. Biol. Chem. **73:** 627-650, 1929.

A FLUOROMETRIC METHOD FOR THE ESTIMATION OF TYROSINE IN PLASMA AND TISSUES

T. P. Waalkes, Ph.D., M.D., and S. Udenfriend, Ph.D.
Bethesda, Md.

THE reaction between 1-nitroso-2-naphthol and tyrosine, under suitable conditions, yields a yellow product which has been utilized for the colorimetric estimation of tyrosine in biologic material. It has now been found that the nitrosonaphthol derivative of tyrosine fluoresces. Measurement of the fluorescence of this derivative endows the procedure of Udenfriend and Cooper[1] with greater sensitivity and specificity than was obtained in their original colorimetric method.

In this report, the fluorometric method is used to determine the amount of tyrosine in plasma from fasting patients with different disease states, as compared to the amount of the amino acid found in the plasma of normal fasting controls. A few of the patients selected had diseases in which it was thought that an alteration in tyrosine metabolism might occur. The survey is not intended as a critical analysis of tyrosine plasma levels, but rather to demonstrate the method and its application.

MATERIAL AND METHODS

Reagents.—1-nitroso-2-naphthol: 0.1 per cent 1-nitroso-2-naphthol (practical grade) in 95 per cent alcohol.

Nitric acid reagent: 24.5 ml. of 1:5 nitric acid is mixed with 0.5 ml. of 2.5 per cent $NaNO_2$.

Procedure.—The chemical method is the same as that previously reported. (1) One milliliter of plasma is diluted to 4 ml. with water, and 1 ml. of 30 per cent trichloroacetic acid is added. After ten minutes, the mixture is centrifuged. To 2 ml. of the deproteinized plasma, in a glass-stoppered centrifuge tube, are added 1 ml. of the nitrosonaphthol reagent, and 1 ml. of the nitric acid reagent. The tube is stoppered, shaken, and placed in a water bath at 55° C. for thirty minutes. After cooling, 10 ml. of ethylene dichloride is added, and the tube is shaken to extract the unchanged nitrosonaphthol reagent. After the tube is centrifuged, the supernatant aqueous layer is transferred to a cuvette and read in the spectrophotofluorometer. The fluorescence of the tyrosine derivative, resulting from its activation at 460 mμ, is measured at 570 mμ.

Plasma was obtained from the blood of patients and of normal individuals who had fasted overnight but had not had any other dietary restrictions. Heparin was added as an anticoagulant. Duplicate plasma samples and a tyrosine recovery sample were run through the entire procedure with tyrosine standards and a reagent blank.

The Aminco-Bowman spectrophotofluorometer was used for measurement of fluorescence and for recording of activation and fluorescence spectra.

RESULTS

In Table I are shown the actual readings obtained on the spectrophotofluorometer at the fluorescence peak of the tyrosine-nitrosonaphthol derivative. Values are shown for tyrosine standards, plasma, and for samples of

From the National Heart Institute, National Institutes of Health, Bethesda, Md.
Received for publication April 15, 1957.

TABLE I. DIRECT READINGS TAKEN FROM THE SPECTROFLUOROMETER FOR THE NITROSONAPHTHOL DERIVATIVE OF TYROSINE IN A PLASMA SAMPLE, STANDARD TYROSINE, AND TYROSINE ADDED TO THE PLASMA

SAMPLE	FLUOROMETER READING		TYROSINE RECOVERY* (PER CENT)
	DUPLICATES	AVERAGE	
Plasma†	51		
	51	51	---
Plasma + 5 γ tyrosine	68		
	70	69	100
Plasma + 10 γ tryosine	86		
	84	85	98
Plasma + 15 γ tyrosine	96		
	98	97	91
5 γ tyrosine	18		
	18	18	---
10 γ tyrosine	35		
	37	36	---
15 γ tyrosine	56		
	56	56	---

*This is calculated on the basis of the expected reading, obtained by adding together the individual readings for plasma and the tyrosine standard.
†One milliliter of plasma was taken for assay.

TABLE II. FASTING PLASMA TYROSINE LEVELS IN NORMAL PATIENTS AND IN PATIENTS WITH VARIOUS DISORDERS

DIAGNOSIS	PATIENT NUMBER	TYROSINE (γ/ML. OF PLASMA)
Normal adult	1	12.1
	2	12.4
	3	11.6
	4	11.9
	5	10.3
	6	12.9
	7	13.0
Essential hypertension	8	11.9
	9	14.8
	10	9.9
Hypertension, congestive heart failure, mild diabetes	11	12.2
Hypertension, obesity	12	13.1
Arteriosclerotic heart disease	13	15.0
	14	15.2
Rheumatoid arthritis	15	11.2
	16	11.6
	17	11.0
	18	8.3
	19	13.1
	20	10.1
	21	9.0
Rheumatoid spondylitis	22	8.3
	23	13.1
Lupus erythematosus	24	9.9
Pheochromocytoma	25	8.0
		8.4*
		12.3†
Malignant carcinoid	26	9.7
	27	14.7
Hyperthyroidism	28	14.2
		13.2*
Malignant melanoma	29	10.3
	30	6.8
		9.5*

*Repeat analysis on a sample obtained at a later date.
†Repeat analysis on a sample obtained after removal of the tumor.

the same plasma to which tyrosine had been added. The recovery of the apparent tyrosine in plasma plus that added ranged from 91 to 100 per cent when compared to the total amount expected. Recoveries of 91 per cent or better, calculated in the same way, were also obtained with all the plasma samples used in the studies shown in Table II.

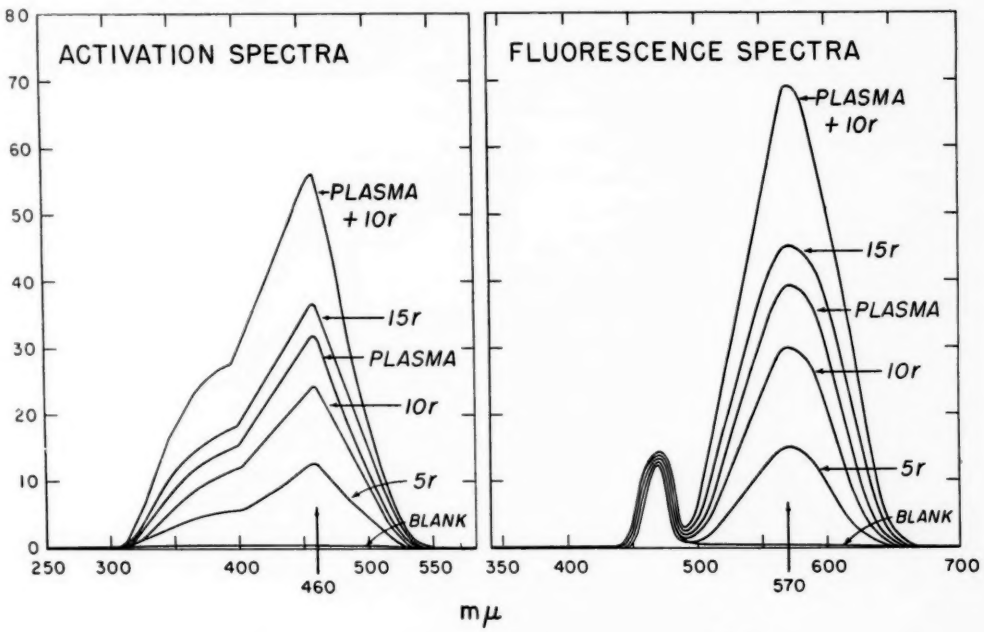

Fig. 1. Fig. 2.

Fig. 1.—Activation spectra for the nitrosonaphthol derivative of tyrosine from (1) standard solutions, (2) plasma alone, and (3) plasma plus 10 γ of tyrosine standard.

Fig. 2.—Fluorescence spectra for the nitrosonaphthol derivative of tyrosine from the identical samples used for Fig. 1.

In Fig. 1 are shown the activation spectra of the tyrosine-nitrosonaphthol derivative from standard tyrosine solutions, from plasma alone, and from the same plasma to which 10 γ of tyrosine standard had been added. Fig. 2 shows the fluorescence spectra for the samples used in Fig. 1. It is apparent that the nitrosonaphthol derivative from plasma has fluorescence characteristics identical with authentic tyrosine, being activated at 460 mμ and fluorescing at 570 mμ.

Fasting plasma tyrosine levels in patients with specific diseases and in seven normal controls are shown in Table II. Levels in the seven normal individuals ranged from 10 to 13 γ per milliliter of plasma. A few of the patients exhibited levels above or below this range.

DISCUSSION

Fluorometric methods of analysis are of value because of their great sensitivity as compared to other methods of assay of biologic materials. Using the

recently developed spectrophotofluorometer,[2] the sensitivity of the nitrosonaphthol method for tyrosine, as described here, is greatly increased in comparison to its colorimetric assay. For example, to read the samples in the plasma filtrates (10 to 15 γ) the spectrophotofluorometer was employed at a sensitivity setting which is actually a fraction of what could be utilized. It has been found that 1 γ or less of tyrosine can be measured by the procedure outlined in this method. In comparison, for a sample containing 10 to 15 γ of tyrosine, the optical density obtained in the Beckman spectrophotometer is only about 0.050, near the limits of its useful range. Moreover, the specificity of the fluorometric method is demonstrated by the similarity of the activation and fluorescence spectra of the nitrosonaphthol derivatives obtained with plasma filtrates and with authentic tyrosine. In contrast, the absorption spectrum of the nitrosonaphthol chromophore from plasma differs considerably from that of the standard tyrosine derivative.[1] The colorimetric method, as applied to plasma, is not specific although it has been shown to be more specific than other chemical methods for tyrosine assay.

The fluorometric method does not distinguish between tyramine and tyrosine; nor does the colorimetric assay. However, the amount of tyramine normally present in tissues is negligible, if present at all, and none is found in plasma.

Although some of the values for plasma from patients with specific diseases were above or below the normal range (10 to 13 γ per milliliter), an insufficient number of determinations was done to make any conclusions from the data. The values for tyrosine in human plasma found here agree well with those previously reported using a microbiologic method.[3]

The method can probably be applied to other biologic materials in addition to plasma. It should be particularly useful in studies where great sensitivity is required.

SUMMARY

A fluorometric method for tyrosine assay has been described. The specificity and sensitivity of the method are discussed, and its application to the determination of tyrosine levels in human plasma has been shown.

REFERENCES

1. Udenfriend, S., and Cooper, J. R.: The Chemical Estimation of Tyrosine and Tyramine, J. Biol. Chem. **196**: 227-233, 1952.
2. Bowman, R. L., Caulfield, P. A., and Udenfriend, S.: Spectrophotofluorometric Assay in the Visible and Ultraviolet, Science **122**: 32-33, 1955.
3. Borden, A. L., Brodie, E. C., Wallraff, E. B., Holbrook, W. P., Hill, D. F., Stephens, C. A. L., Johnson, R. B., and Kemmerer, A. R.: Amino Acid Studies and Clinical Findings in Normal Adults and Rheumatoid Arthritis Patients Treated With ACTH, J. Clin. Invest. **31**: 375-379, 1952.

ESTIMATION OF URINARY CATECHOL AMINES BY MEANS OF A STRIP OF RABBIT AORTA AS AN AID IN THE DIAGNOSIS OF PHEOCHROMOCYTOMA

OSCAR M. HELMER, PH.D.
INDIANAPOLIS, IND.
WITH THE TECHNICAL ASSISTANCE OF R. M. SANDERS

AN INCREASED urinary output of epinephrine and norepinephrine in pheochromocytoma has been verified both by bio-assay[1-3] and by chemical methods.[4] We have found, as have Furchgott and Bhadrakom,[5] that spirally cut strips of thoracic aorta of rabbits are admirably suited for the qualitative and quantitative assay of epinephrine and norepinephrine. Consequently, when asked to perform an assay for catechol amines on urine from a patient suspected of having a pheochromocytoma, urine was tested on the aortic strip. When 0.2 ml. of unfractionated urine was added to the muscle chamber, a maximal contraction occurred. An equivalent amount of urine from a normotensive individual caused only an insignificant shortening. From this response, it appeared that this technique might be useful for screening patients with hypertension for the possible presence of a pheochromocytoma. In this report the experience with this technique is presented.

MATERIALS AND METHODS

Spirally cut strips of rabbit thoracic aorta are prepared as described by Furchgott and Bhadrakom.[5] Rabbits weighing 4 to 6 pounds are killed by a blow on the head, and bled by severing the blood vessels in the neck. The descending thoracic aorta is removed and placed in a Petri dish containing Krebs bicarbonate solution[6] at room temperature. Excess fat and connective tissue are trimmed off. The whole length of aorta is then cut along a close spiral. Strips of approximately 40 to 45 mm. in length (unstretched) are used for the assay. The strips are mounted in a muscle chamber of 20 ml. capacity with the aid of stainless steel hooks. The muscle chamber assembly is illustrated in Fig. 1. The bath is maintained at 37.5° C. The strip is bathed with Krebs bicarbonate solution containing 0.2 per cent glucose. Through this solution, both in the muscle chamber and reservoir bottle, 95 per cent O_2 and 5 per cent CO_2 is bubbled to maintain the pH of 7.4. The isotonic lever is adjusted to give a ninefold amplification and counterweighted to exert 4 Gm. tension on the strips. The contraction is recorded on "Waxer" paper (Gorrell and Gorrell) by means of a heated stylus attached to the lever. A small vibrating motor is clamped to the rod holding the lever to eliminate any friction of the stylus on the paper.

Collection and Preservation of Urine.—Immediately after the urine has been voided, it is adjusted to pH 3.0 by the addition of HCl to prevent inactivation of the catechol amines. Von Euler[7] has found that even after 15 days at room temperature at pH 1 to 3 catechol amines in urine are not appreciably inactivated. We have found that when kept in a frozen state, catechol amines in urine at pH below 3 maintain activity for over a year.

From the Lilly Laboratory for Clinical Research, Indianapolis General Hospital, Indianapolis, Ind.
Presented in part before the Annual Meeting of the Central Society for Clinical Research, Nov. 4 and 5, 1955, Chicago, Ill.
Received for publication April 25, 1957.

Since it is sometimes difficult to obtain 24-hour urine specimens in an open ward or outpatient department, the small amount of urine needed for assay with the aortic strip method makes it possible to collect urine over a shorter period. Most of the assays reported in this paper have been done on urine collected over a 2-hour period. If the samples are not assayed at once, an aliquot is stored in a frozen state.

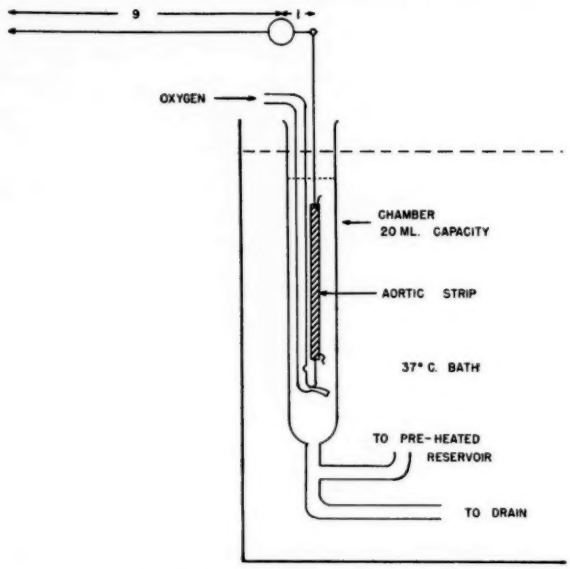

Fig. 1.—Muscle chamber assembly for aortic strip.

Assay Procedure.—The strip is not used for assays until maximal sensitivity to norepinephrine or epinephrine is attained. This usually occurs after 2 or 3 additions of 0.05 to 0.1 μg of norepinephrine to the chamber. With these doses, contractions of 21 to 44 mm. are obtained. The time required for the strip to reach maximal responsiveness is about 1½ hours.

In order to compensate for differences in urine volume, an amount of unfractionated urine equivalent to that excreted in 0.1 to 0.2 minute is added directly to the muscle chamber from a tuberculin syringe fitted with a needle long enough to dip below the surface of the solution in the chamber. The plunger is moved back and forth 3 times to insure proper mixing.

Test substances are washed out of the chamber within 1 to 2 minutes after maximal contraction is attained.

RESULTS

Qualitative Assay.—In Fig. 2 is shown an example of an assay of urine for catechol amines. Unfractionated urine equivalent to that excreted in 0.1 minute was added to the muscle chamber. The specimen from a patient suspected of having pheochromocytoma caused a strong contraction; whereas an equivalent amount of urine from a normotensive subject or from a patient with essential hypertension produced no shortening of the muscle strip. The specificity of the test was shown by the addition of 0.1 mg. Regitine (phentolamine methanesulfonate) to the muscle chamber and the retesting of the urine from the patient suspected of having pheochromocytoma. In the presence of Regitine, this urine was inactive. The strip was also unresponsive to norepinephrine.

Since the constrictor activity of angiotonin is not abolished by adrenolytic agents, angiotonin was added to the muscle chamber in order to test the viability of the strip. The resulting contraction indicated that Regitine did not have an injurious effect.

Since normal urine contains hydroxytyramine in addition to epinephrine and norepinephrine (von Euler, Hamberg, and Hellner[8]), the possibility that this catechol amine might influence the assay had to be considered. Hydroxytyramine behaves like epinephrine and norepinephrine in two respects. First, a factor in urine enhances the ability of each of these agents to cause a contraction of the strip; second, Regitine also inactivates it. However, hydroxytyramine is only about one-fiftieth as active as norepinephrine on the rabbit aorta strip and should have little effect on the assay.

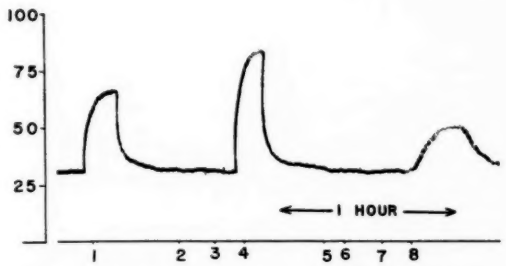

Fig. 2.—Assay of unfractionated urines for catechol amines on strip of rabbit aorta. 1, 0.5 mg. Versene + 0.15 μg norepinephrine; 2, normal urine equivalent to 0.1 minute; 3, urine from a patient with essential hypertension equivalent to 0.1 minute; 4, pheochromocytoma urine equivalent to 0.1 minute; 5, Regitine (100 μg); 6, pheochromocytoma urine equivalent to 0.1 minute; 7, 0.5 mg. EDTA + 0.15 μg nonepinephrine; 8, 0.5 unit angiotonin. Ordinate, record of contraction in millimeters. Lever amplifies contraction 9 times.

Additional evidence that hydroxytyramine does not interfere with the assay was obtained by the use of Marsilid (iproniazid*), an amine oxidase inhibitor. This agent markedly potentiates the contraction produced by both tyramine and hydroxytyramine. It has only a slight effect on epinephrine or norepinephrine on the strip. The addition of Marsilid to the muscle chamber has not enhanced the contraction produced by either normal urine or urine from patients with pheochromocytoma so far tested.

Weil-Malherbe[9] recently described a patient with pheochromocytoma in which the urinary excretion of hydroxytyramine was increased as much as, or more than, the excretion of noradrenaline. The addition of Marsilid to the bath would be useful in detecting such cases.

Further indications of the specificity of the test are demonstrated in Fig. 3, in which a urine from a patient with pheochromocytoma was assayed before and after the removal of the tumor. The preoperative urine specimen caused a strong contraction; the postoperative sample gave the same response as a urine from a normotensive subject. Identical results were obtained with urines collected before and after operation from 4 other patients.

*I wish to thank Dr. Elmer L. Sevringhaus of Hoffmann-La Roche, Inc., for kindly supplying the Marsilid (iproniazid) used in this study.

Quantitative Assay of Pheochromocytoma Urine.—For screening hypertensive patients for the possible presence of a pheochromocytoma, a quantitative assay is not in most instances necessary. The difference between the contraction produced by urine from patients with essential hypertension and proved cases of pheochromocytoma that have been assayed in this laboratory has been obvious. The amount of urine equivalent to 0.1 to 0.2 minute of the former group of patients causes only an insignificant contraction, whereas with the latter group, an amount of urine equivalent to 0.02 to 0.1 minute has caused a strong contraction. However, between the two extremes are a group of patients whose urine causes a shortening of the muscle strip that is definitely

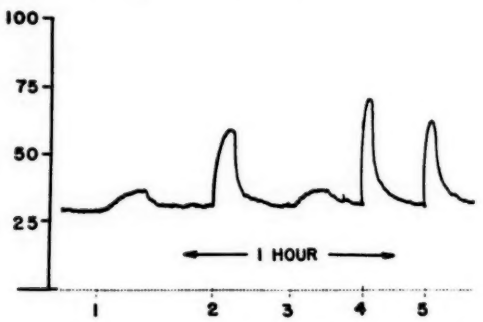

Fig. 3.—Contraction of aortic strip induced by urine from a patient with pheochromocytoma before and after removal of tumor. 1, Normal urine equivalent to 0.1 minute; 2, preoperative urine equivalent to 0.1 minute; 3, postoperative urine equivalent to 0.1 minute; 4, 0.3 μg norepinephrine; 5, 0.2 μg norepinephrine.

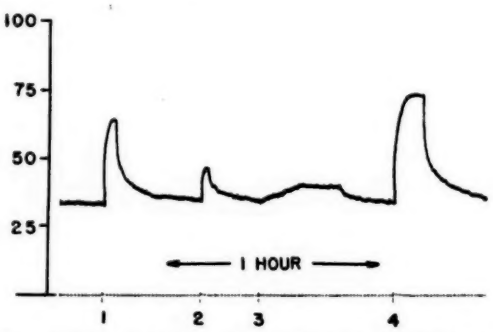

Fig. 4.—Ability of urine to enhance contraction produced by epinephrine. 1, 0.1 μg epinephrine; 2, 0.05 μg of epinephrine; 3, 0.1 ml. normal urine; 4, 0.1 ml. normal urine + 0.05 μg epinephrine.

more than that produced by average normal urine but not as much as that from proved cases of pheochromocytoma that we had examined early in our studies. In order to make a better interpretation of the results of the assay of such urines, a quantitative assay has been done on all urines that cause a definitely greater contraction than normal urine, as well as urines from a group of normotensive subjects.

There is a factor in urine which greatly enhances the contraction produced by epinephrine and norepinephrine (Fig. 4). This enhancement is similar to that produced by ethylenediaminetetraacetic acid (EDTA). Therefore, the

catechol amine content of urine cannot be assayed directly against these constrictor agents. In the quantitative assay of urines from patients with pheochromocytoma, norepinephrine with the addition of 0.5 mg. of EDTA to the bath has been used as a standard. It is also possible to substitute for the EDTA an amount of normal urine equivalent to that of the pheochromocytoma urine being assayed. The low catechol amine content of the normal urine would not interfere in assays of urines from patients with pheochromocytoma with high catechol amine values.

In Fig. 5 is shown an example of a quantitative assay of a urine from a pheochromocytoma patient. In this instance the urine being assayed was bracketed by known concentrations of norepinephrine; in other cases an exact match of the unknown and the standard was achieved.

In order to check the validity of the quantitative assay of urines which had a high catechol amine content, such urines were injected intravenously in a pithed cat to compare their pressor activity to that of norepinephrine.

Fig. 5.—Quantitative assay of urine from patient with pheochromocytoma. 1, 0.5 mg. EDTA + 0.2 μg norepinephrine; 2, 0.04 ml. of pheochromocytoma urine; 3, 0.5 mg. EDTA + 0.25 μg of norepinephrine. Calculated value—5.6 μg per ml.

In Table I is shown a comparison of the aortic strip and pressor assay of 3 urines, 2 of high catechol amine content, and 1 which is definitely higher than normal but not as high as the proved cases of pheochromocytoma in our series. The assays of the more active urines were in good agreement by both methods. For the assay of these urines in a cat, amounts of urine equivalent to only 0.17 to 0.27 minute were required to produce rises in arterial blood pressure of 38 to 24 mm. Hg.

TABLE I. COMPARISON OF THE AORTIC STRIP TECHNIQUE WITH DIRECT PRESSOR ASSAY IN CATS FOR THE DETERMINATION OF THE CATECHOL AMINE* CONTENT OF UNFRACTIONATED URINE

PATIENT	STRIP ASSAY (μG/ML.)	PRESSOR ASSAY (μG/ML.)
M. C.	5.7	5.0
M. S.	0.8	0.9
H. T.	1.0	0.27

*Expressed as norepinephrine.

With the urines of a lower catechol amine content, the pressor assay gave a much lower value than that obtained on the strip. An amount of urine equivalent to 1.85 minutes gave only a 14 mm. rise in blood pressure. With the larger

amount of urine required, the lower value obtained in the pressor assay is probably due to the presence of kallikrein, an enzyme in urine which acts on the alpha-2 globulin fraction of plasma to form a depressor polypeptide, kallidin.[10]

Assays have been done on 560 urines from patients with elevated arterial blood pressure. Many of the urines were sent by mail from hospitals in other cities. In Table II are recorded the results of the quantitative assay of urines of 10 patients in whom the presence of pheochromocytoma has been proved by operation in 9 and by post mortem in 1. In 9 of these patients, the catechol amine content was more than 18 μg per hour. The highest value found was 396 μg per hour. One patient of the paroxysmal type, who had a positive histamine test, excreted only 8 μg per hour. The blood pressure of this patient at the time of urine collection was not recorded. After the removal of the tumor, only about 1 to 2 μg of catechol amines per hour were found in the urine.

TABLE II. URINARY EXCRETION OF CATECHOL AMINES, EXPRESSED AS NOREPINEPHRINE OF PATIENTS IN WHOM THE PRESENCE OR ABSENCE OF A PHEOCHROMOCYTOMA WAS VERIFIED BY OPERATION

PATIENT	μG/HR.	TUMOR + OR −	PHARMACOLOGICAL TESTS
M. C.	396	+	Regitine +
P. D. Y.	302	+	Not done
D. M.	144	+	Regitine +, Histamine −
M. S.	107	+	Regitine +
J. E.	98	+	Not done
F. G.	96	+	Regitine +
J. Sh.	37	+	Regitine +
M. B.	30	+	Not done
A. N.	18	+	Regitine +
P. B.	15-22	−	Regitine +
R. K.	15	−	Regitine +
A. F.	8	+	Histamine +

In addition to these patients 3 others were found who excreted 33 to 63 μg of catechol amines per hour. At this time these patients have not been subjected to an operation.

The average value of urinary catechol amines in 11 healthy laboratory workers was found to be 3.6 μg per hour with a range of 1.6 to 6.6 μg. Between these figures and the definitely elevated values found in the proved cases of pheochromocytoma during a phase of elevated pressure was a group of 6 patients with catechol amine concentrations in the urine which ranged from 12 to 22 μg per hour. Surgery was performed on 2 of these patients; in neither was an adrenal tumor found.

One of the patients (R. K.) had a blood pressure which varied between 110/70 and 180/120; she excreted 15 μg of catechol amines in 2 samples of urine submitted 2 months apart. When Regitine was given to her at a pressure of 180/120, there was a fall in blood pressure to 120/70. This patient weighed 235 pounds. Another patient (P. B.) with a fixed elevation of pressure, who had a positive Regitine test on two occasions, had urinary catechol amine values ranging from 15 to 22 μg in 3 urine samples. A tumor was not found in this patient on exploration of the abdominal cavity.

Five patients who did not have an elevated catechol amine content in their urine have been subjected to operation or have had a post-mortem examination. In none of these patients was a pheochromocytoma found.

The value of adding Regitine to the bath to test whether the contraction produced by a urine being assayed is due to a catechol amine was demonstrated conclusively in a specimen obtained from a diabetic patient. Two specimens of urine which were mailed to this laboratory caused a strong contraction of the strip. The contraction was not abolished by Regitine.* A kidney extract containing cathepsins destroyed the constrictor substance in this urine, indicating the presence of a polypeptide, perhaps kallidin, which stimulates smooth muscle preparations.[10]

The Effect of Therapeutic Agents on Assay.—Chlorpromazine, hydralazine, and adrenolytic agents, when added to the aortic strip, will prevent the constriction produced by epinephrine and norepinephrine. Consequently, if patients are receiving these medications, there is a possibility that the excretion of these substances in the urine might interfere with the assay. Two urines that were assayed abolished the contraction produced by norepinephrine. One of the patients was receiving hydralazine. Information was not received on the therapy of the other patient. The urine collected 4½ hours after a Regitine test inhibited norepinephrine; that collected after 7½ hours had no such effect.

Urine obtained from patients treated with reserpine, ganglionic blocking agents, or barbiturates did not interfere with the assay.

DISCUSSION

The estimation of catechol amines in urines by means of a spirally cut strip of rabbit aorta appears to be a useful adjunct in the diagnosis of pheochromocytoma. It has these advantages. Since such small amounts of unfractionated urine are required for the assay, only a 1- to 2-hour urine sample need be collected. If urines are adjusted to pH 3.0 or below, they can be stored in a refrigerator or in a frozen state until it is convenient to assay them. The method is rapid. With one strip, urines of normal catechol amine content can be screened at the rate of 5 to 6 an hour. The method has also proved useful as a confirmatory test in the diagnosis of pheochromocytoma.

In most of the proved cases of pheochromocytoma reported in this paper, the catechol amine content of the urine was so high a quantitative assay was not necessary. With values of 20 μg per hour or higher, false positives would be unlikely.

On the other hand, it is quite possible that in patients of the paroxysmal type, an elevated content of catechol amines might not be found, particularly between attacks. This possibility is emphasized by Litchfield and Peart on the basis of an interesting case they described recently.[11] These authors concluded from the findings in this case that although the assay of catechol amines in

*The urine from this patient was also sent to Dr. Marcel Goldenberg for a chemical assay for catechol amines. This laboratory did not find an elevated content of epinephrine or norepinephrine.

the urine is probably the most reliable test for confirming the diagnosis of pheochromocytoma, proper caution should be exercised particularly in patients with paroxysmal hypertension. Between attacks the urinary catechol amines can be normal or only slightly elevated. In Litchfield and Peart's case, even after an attack, it was difficult for them to say that a significant increase in urinary pressor amines occurred. They feel that it is necessary to abide by the clinical diagnosis especially if a typical attack of hypertension with pallor is observed. A provocative histamine test should prove very helpful in such cases.

Patient A. F. in our series represents a similar situation. The urinary catechol amine value of 8 μg per hour would not be high enough over that found in some healthy individuals to warrant the diagnosis of pheochromocytoma. In this case, the positive histamine test and clinical picture were more definitive. After the operation, catechol amine value was reduced to about one-fifth the preoperative value, a spread as great as that reported for normal individuals.

SUMMARY

The use of a muscle bath, employing a spirally cut strip of rabbit aorta for the determination of catechol amines in the urine has been described. It has proved to be useful for screening patients with hypertension for the presence of a pheochromocytoma. Only small amounts of unfractionated urine, equivalent to that excreted in 0.1 to 0.2 minute, are required for the assay.

I wish to thank the many physicians who sent us urines for assay and Miss Helen Wagoner for help in preparing the manuscript.

REFERENCES

1. Engel, A., and von Euler, U. S.: Diagnostic Value of Increased Urinary Output of Noradrenaline and Adrenaline in Phaeochromocytoma, Lancet **259**: 387, 1950.
2. Goldenberg, M., and Rapport, M. M.: Nor-epinephrine and Epinephrine in Human Urine (Addison's Disease, Essential Hypertension, Pheochromocytoma), J. Clin. Invest. **30**: 641-642, 1951.
3. von Euler, U. S.: Increased Urinary Excretion of Noradrenaline and Adrenaline in Cases of Pheochromocytoma, Ann. Surg. **134**: 929-933, 1951.
4. Goldenberg, M., Serlin, I., Edwards, T., and Rapport, M. M.: Chemical Screening Methods for the Diagnosis of Pheochromocytoma. I. Nor-epinephrine and Epinephrine in Human Urine, Am. J. Med. **16**: 310-327, 1954.
5. Furchgott, R. F., and Bhadrakom, S.: Reactions of Strips of Rabbit Aorta to Epinephrine, Isopropylarterenol, Sodium Nitrite and Other Drugs, J. Pharm. & Exper. Therap. **108**: 129-143, 1953.
6. Krebs, H. A., and Henseleit, K.: Untersuchungen über die Harnstoff-bildung im Tierkörper, Ztschr. physiol. Chem. **210**: 33-66, 1932.
7. von Euler, U. S.: Noradrenaline, Springfield, Ill., 1956, Charles C Thomas.
8. von Euler, U. S., Hamberg, U., and Hellner, S.: β-(3:4-Dihyroxyphenyl) Ethylamine (Hydroxytyramine) in Normal Urine, Biochem. J. **49**: 655-658, 1951.
9. Weil-Malherbe, H.: Phaeochromocytoma—Catechols in Urine and Tumor Tissue, Lancet **271**: 282-284, 1956.
10. Werle, R.: The Chemistry and Pharmacology of Kallikrein and Kallidin, Polypeptides Which Stimulate Plain Muscle, edited J. H. Gaddum, Edinburgh and London, 1955, E. & S. Livingstone, Ltd.
11. Litchfield, J. W., and Peart, W. S.: Pheochromocytoma With Normal Excretion of Adrenaline and Noradrenaline, Lancet **271**: 1283-1284, 1956.

A NEW TECHNIQUE FOR DIFFERENTIATION OF HEMOGLOBIN

ABNER R. ROBINSON, M.S., MARGARET ROBSON, B.S., ANN P. HARRISON, B.S., AND WOLF W. ZUELZER, M.D.
DETROIT, MICH.

THE detection of small amounts of a hemoglobin component in the presence of large amounts of another hemoglobin by means of paper electrophoresis is difficult. This problem has been particularly evident with respect to the determination of fetal hemoglobin. Chemically, the Singer 1-minute alkali resistance method[1] is unreliable at low levels; the more refined techniques are laborious and may give erroneous results in the presence of carbon monoxide hemoglobin.[2] Also, the identification of alkali resistant hemoglobin by free electrophoresis is difficult and requires considerable care.

The long known but little used agar gel electrophoresis medium seemed to us to offer some hope of separating the fetal component. Gordon and associates[3] have demonstrated the separation of hemoglobin from ferritin, and Bussard and Perrin[4] have used agar as a supporting medium for serum protein separation. The present method was first devised with the intent of providing an easy, clear-cut method of separating fetal (alkali resistant) hemoglobin. However, during the course of its development it proved to be valuable in detecting and quantitating both alkali resistant and other hemoglobins. The method requires very little additional care than paper electrophoresis in operation and when used in conjunction with paper electrophoresis provides a means of distinguishing between hemoglobin D and hemoglobin S.

The method has been applied to artificial and naturally occurring mixtures of hemoglobins A, F, S, D, and C. The components A, F, S, and C gave characteristic separations (Fig. 1), whereas D did not separate from A (Fig. 5).

METHODS AND MATERIALS

Hemoglobin Solutions.—Hemoglobin solutions were prepared by washing 3 times with isotonic saline, and lysing with distilled water and toluene. The solutions were allowed to stand overnight in the refrigerator and then centrifuged at 3,000 r.p.m. The clear hemolysate was then removed with a needle and syringe. All samples were run as oxyhemoglobin. The carboxyhemoglobin was first used but no difference in the pattern could be detected from that obtained with oxyhemoglobin samples.

Paper Electrophoresis.—The paper patterns were obtained using a veronal buffer, pH 8.6, ionic strength 0.05, in the apparatus described by Zuelzer and associates.[5] Time of run was 16 hours at 7 Ma. and 250 volts.

Exchange Chromatography.—The fetal hemoglobin preparation was carried out using IRC 50 resin and a citric acid-citrate buffer as described in the method of Prins and Huisman.[6]

From the Child Research Center of Michigan, Detroit, Mich.
This investigation was supported in part by a grant from the Rockefeller Foundation.
Received for publication May 1, 1957.

Solubility.—The solubility determinations were made using the method of Itano.[7]

Agar Electrophoresis.—The separation of hemoglobin using agar as a media was achieved as follows: a 1 per cent agar solution* was prepared using a citric acid-sodium citrate buffer, pH 6.5 and 0.05 M. (The buffer was prepared by dissolving 147 Gm. of sodium citrate [2 H_2O] in 600 ml. of H_2O, adjusting to pH 6.0 with concentrated citric acid solution, and diluting to a liter. This was the stock buffer solution. The buffer was prepared for use by diluting the stock solution, 1 part stock solution plus 9 parts water, and adjusting to pH 6.2 with citric acid solution.) The agar was heated until clear and then allowed to cool until just above the gelling point and then poured on a 4 by 10 inch thin glass plate which had filter paper wicks placed on each end and allowed to gel (approximately 25 ml. of solution is adequate). When the agar had cooled to room temperature samples of hemoglobin solution were introduced in the gel in the following manner: slits approximately ¾ inch long were made in the gel across the end of the plate with ¼ inch between the slits. A small strip of filter paper was then soaked with hemoglobin solution of approximately 10 Gm. per 100 ml. concentration and inserted in the slits. The entire plate was coated with a plastic by spraying with Krylon.† This is essential to prevent drying of agar during electrophoresis.

The plate was placed in a paper electrophoresis apparatus previously described[5] which contained the same buffer as used to make up the agar solution. A current of 20 Ma. and 350 volts (supplied by a Heath Kit) produced good separation in 16 hours.

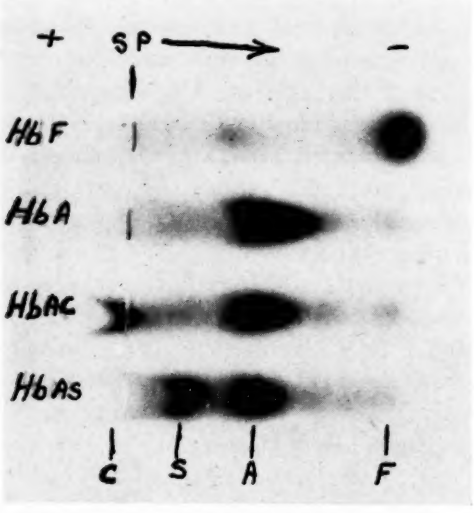

Fig. 1.—Typical agar plate pattern, with samples of hemoglobins F, A, AC, and AS.

The hemoglobins under these conditions migrate toward the cathode as would be expected at this pH. However, as can be seen in Fig. 1, the order of separation is not the same as that found in free electrophoresis at pH 6.2, since alkali resistant hemoglobin migrates fastest with hemoglobins A, S, and C following in that sequence. This is the reverse of the order found in free electrophoresis but very similar to the manner in which hemoglobins separate on the IRC 50 resin column of Prins and Huisman.[6] This order would indicate that the separations are due to a combination of adsorption and electrophoretic effects.

The agar plates may be stained by stripping off the plastic coating and staining with Amino-Schwartz 10B in a methyl alcohol, water, and acetic acid solution (50:50:5) for 20 minutes followed by careful washing with 2 per cent acetic acid solution. After staining

*Bacti-Agar, Difco Laboratories, Detroit, Mich.
†Manufactured by Krylon, Inc., Norristown, Pa.

and washing, the plates are dried at room temperature. The agar adheres to the plate like photographic emulsion and gives a low background when read in a densitometer. Quantitation by this means yields results comparable to that obtained on paper.

RESULTS

Fig. 1 illustrates the typical separations obtained using hemolysates from known samples of F, A, AC, and AS hemoglobins. The F sample (cord blood)

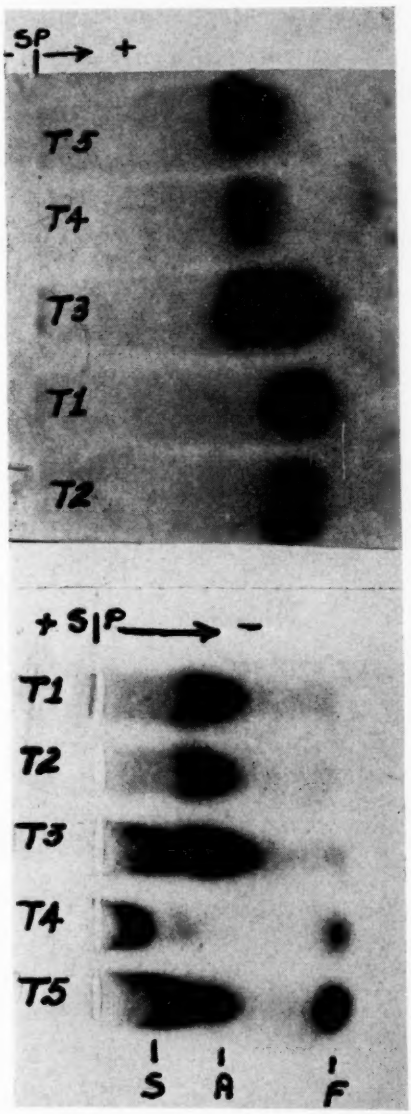

Fig. 2.—Comparison of (*A*) paper electrophoresis, and (*B*) agar electrophoresis in a family study of sickle-cell thalassemia.

had been found to have 87 per cent alkali resistant hemoglobin. As can be clearly seen, the pattern of the F sample demonstrates the presence of a small amount of A which paper electrophoresis had failed to show. The A, AC, and

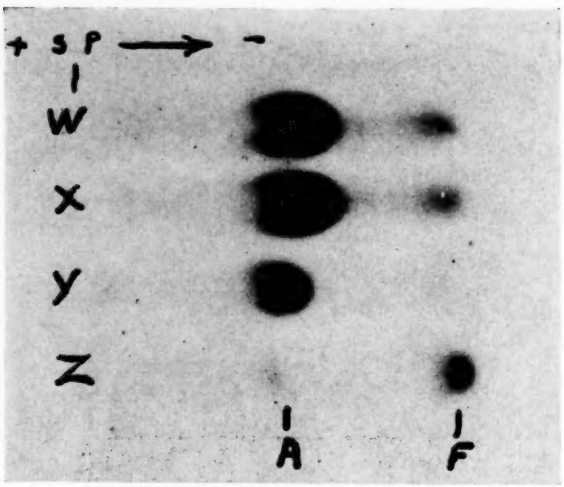

Fig. 3.—Illustration of effect of decreasing amount of hemoglobin introduced on agar plate.

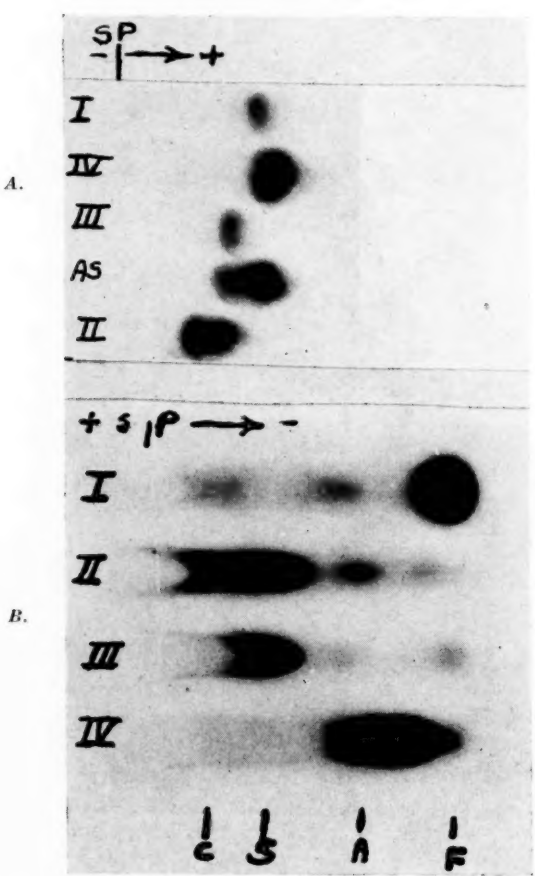

Fig. 4.—Comparison of small components using (*A*) paper electrophoresis, and (*B*) agar plate method.

AS samples contained between 1 and 2 per cent alkali resistant hemoglobin. As can be seen in the illustration, A, S, and C are clearly separated and a spot appears in the F position with an intensity comparable to the percentage of alkali resistance obtained by the chemical method.

A second faint spot, visible on agar in the A, AC, and AS samples just ahead of the A position, has not been identified as yet but was present in many samples examined. In the A and AC patterns there was also an increased density in the S position. This was reproducible and occurred in only a few specimens. The authors have as yet been unable to determine whether this represents sickle hemoglobin, another hemoglobin with a mobility similar to sickle, or an artifact. However with the increasing reports in the literature[8, 9] of minor components in normal hemoglobins, these small amounts of hemoglobin or hemoglobin-like substances merit attention.

Fig. 2 demonstrates the use of the agar method in a family with sickle thalassemia disease in two members.

Case Report.—T4 was a 12-year-old boy whose blood sickled on smear and who had a large number of target cells. His hemoglobin was 2 Gm. per 100 ml. and his R.B.C. was 800,000.

T5 was a 16-year-old girl whose blood sickled on smear and had a large number of target cells. Her hemoglobin was 9 Gm. per 100 ml. and her R.B.C. was 4,100,000, hematocrit 31 per cent, and reticulocytes 2.2 per cent.

The blood of the father (T3) was that of a typical sickle trait; the blood of the mother (T2) manifested thalassemia minor.

Comparing agar patterns with paper patterns it was seen that T4 and T5 on paper showed predominantly S with only an indication of F, while on agar T5 clearly showed A and F, and T4 showed a faint indication of A with a distinct spot for F. The hemoglobin F is very clearly separated. Chemically T5 had 10 per cent, and T4 15 per cent alkali resistant hemoglobin. This may not appear so from the illustration, but the T4 hemolysate was very weak (3 Gm. per 100 ml.) as compared to T5 whose hemolysate was 10 Gm. per 100 ml. This was unavoidable as T4 was severely anemic. T3, the father, clearly demonstrates the presence of A and S with a small amount of F (1 per cent alkali resistant). The mother (T2) and sibling (T1) possessed only A with less than 1 per cent alkali resistant. Again good agreement existed between the intensity of the F spot and alkali resistance determined chemically.

The concentration of hemoglobin in the hemolysate was found to be significant. Fig. 3 illustrates the effect of lowering the hemolysate concentration. Sample W was A with 2 per cent alkali resistant hemoglobin and an hemolysate of 11 Gm. per 100 ml.; sample X was A with 1.8 per cent alkali resistant hemoglobin with an hemolysate of 6 Gm. per 100 ml.; sample Y was A with 2.9 per cent fetal, hemolysate 3 Gm. per 100 ml.; sample Z was A with 56 per cent alkali resistant hemoglobin and an hemolysate of 2 Gm. per 100 ml.

It can be seen from this illustration that variation of the hemolysate concentration from 6 to 10 Gm. per 100 ml. causes little difficulty in the recognition of minor components. However when the concentration was dropped to

3 Gm. per 100 ml., the F component was not discernible. The necessity of maintaining the hemoglobin concentration at the proper level is more clearly demonstrated in sample Z where the A component is barely discernible. It should also be noted that with increasing amounts of hemoglobin, the spot increases in the direction of movement. Note the difference between the A component in samples Y and X. If the concentration is increased too much or if too large an amount of hemolysate is introduced onto the plate, an "overload" pattern develops in which a single spot migrates from the A to the F position and all intermediate details are obliterated (see Fig. 4, sample IV).

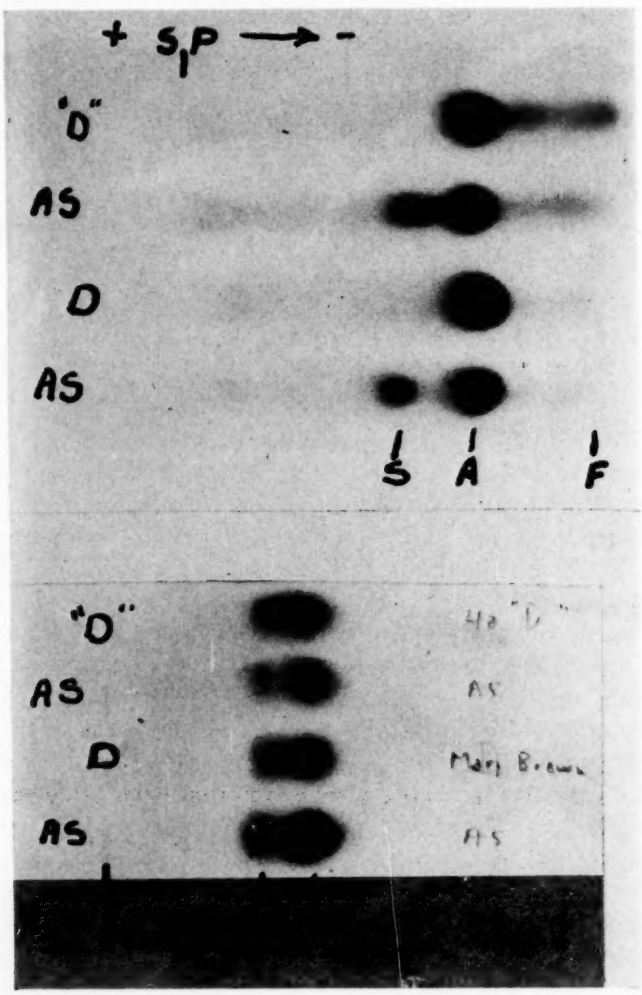

Fig. 5.—Illustration of differences in behavior of hemoglobin D and hemoglobin S on (*A*) agar, and on (*B*) paper electrophoresis.

Fig. 4, sample I, obtained from a newborn infant, showed the presence of A- and C-like components, demonstrated on agar when not detectable on paper. This sample contained 94 per cent alkali resistant hemoglobin when tested by

chemical methods and appeared as a single spot on paper. The confirmation of the actual presence of A and C must await the disappearance of the fetal hemoglobin and the appearance of the adult hemoglobin in this infant.

Sample II was shown on paper to contain S and C, the alkali resistance tested chemically was 1.6 per cent and the sickle preparation was positive. When tested on agar the presence of distinct spots in the A and F positions was noted.

Sample III appeared as a single component, S, on paper. The patient's erythrocytes sickled on smear. The hemoglobin showed 1 per cent alkali resistance. No signs of thalassemia were manifested. On chemical and genetic grounds this individual was thought to have sickle-cell anemia. When sample III was analyzed on agar a faint spot was visible in the A position in addition to a spot in the F position. Thus, it would appear from samples II and III that an A-like component does occur in the SC combination and that apparently pure S may also be accompanied by an A-like component (as in sample III).

Sample IV was hemoglobin A and illustrated what happened when the agar is overloaded with hemoglobin.

Fig. 5 demonstrates the behavior of D. As was clearly shown by the paper patterns, samples D and "D" separated into two spots in the A and S positions. Both samples were likewise shown to have peaks in the A and S positions in free electrophoresis using pH 6.5, 0.1 ionic strength, cacodylate buffer. The samples were tested for solubility by the Itano method[7] and found to have solubilities identical with that of an AD mixture. As can be seen in Fig. 5, on agar a single spot in the A position was found. D, thus, did not separate from A. However, S, in an AS combination, did clearly separate.

This method in conjunction with paper electrophoresis provides a means of identifying D in the presence of S without testing for solubility.

The method has also been employed on samples of hemoglobin AG. It was found that G separated in a position just slightly ahead of S. Unfortunately illustrations of this effect are not available. It is hoped that more samples of the now known types of hemoglobin will become available to us for testing by this technique.

DISCUSSION

The agar technique has been employed for the detection of both small and large amounts of alkali resistant hemoglobin. The concentration detected varied from less than 1 per cent to 94 per cent. The ability of the method to detect very low concentrations of alkali resistant hemoglobin and yet maintain good agreement with chemical determinations make the method a useful tool. In the development of the method, however, its ability to separate major components, and, on the other hand, to show the presence of small components of other hemoglobin-like substances has further increased its value. Indeed in the light of the possible genetic implications, these findings of an A-like substance in the SC combination and in the S homozygote, may be the method's most valuable aspect.

The additional demonstration of the failure of the method to separate D from A provides, when taken in conjunction with paper electrophoresis, a means other than the solubility for demonstrating the presence of this hemoglobin.

SUMMARY

The technique as described here has provided a means of clearly demonstrating the presence of alkali resistant hemoglobin electrophoretically. We are also able to separate hemoglobins A, S, and C in a manner comparable to that obtained by paper electrophoresis. The method is unable to separate D from A but when used in conjunction with paper electrophoresis and the sickling technique thus provides another characterization of D.

At least one other component is demonstrated in normal blood which as yet has not been identified. The appearance of minor components in normal and abnormal blood in the position of known hemoglobins and in intermediate positions awaits further investigation and application of this method.

REFERENCES

1. Singer, K., Chernoff, A., and Singer, L.: Studies on Abnormal Hemoglobins. I. Their Demonstration in Sickle Cell Anemia and Other Hematologic Disorders by Means of Alkali Denaturation, Blood **6**: 413, 1951.
2. Jonxis, J. H. P., and Huisman, T. H. J.: The Detection and Estimation of Fetal Hemoglobin by Means of the Alkali Denaturation Test, Blood **11**: 1009, 1956.
3. Gordon, A. H., Keil, B., Sebasta, K., Knessl, O., and Sorm, F.: Electrophoresis of Proteins in Agar Jelly, Collection Czechoslov. Chem. Communs. **15**: 1, 1950.
4. Bussard, A., and Perrin, D. : Electrophoresis in Agar Plates, J. LAB. & CLIN. MED. **46**: 689, 1955.
5. Zuelzer, W. W., Neel, J. V., and Robinson, A. R.: Abnormal Hemoglobins in: Progress in Hematology, vol. I, New York, 1956, Grune & Stratton, Inc., pp. 103-104.
6. Prins, H. K., and Huisman, T. H. J.: Chromatographic Estimation of Different Kinds of Human Hemoglobin, Nature **175**: 903, 1955.
7. Itano, H. A.: The Solubilities of Naturally Occurring Mixtures of Human Hemoglobin, Arch. Biochem. **47**: 148, 1953.
8. Morrison, M., and Cook, J. L.,: Chromatographic Fractionation of Normal Adult Hemoglobin, Science **122**: 920, 1955.
9. Kunkel, H. C., and Wallenius, G.: New Hemoglobins in Normal Adult Blood, Science **122**: 288, 1955.

THE LABORATORY DETERMINATION OF PEPSIN IN GASTRIC JUICE WITH RADIOACTIVE IODINATED ALBUMIN

ARTHUR P. KLOTZ, M.D.,* AND MELVA R. DUVALL**
KANSAS CITY, KAN.

THE determination of pepsin in gastric juice is not a routine laboratory procedure. Occasionally pepsin determinations have been performed to estimate expected progress in the healing of known peptic ulcer.[1] Little clinical attention, however, is given to gastric pepsin currently since the main attack of ulcer therapy is in neutralization of the free acid in the stomach.

When pepsin determinations are performed in the laboratory today, the hemoglobin method is the procedure most frequently used. This method usually involves diluting the gastric juice sample in order to decrease the pepsin concentration to the sensitivity range of the test. This dilution factor introduces a variable in peptic activity that must be taken into account in estimating the concentration of the enzyme.[2, 3]

A simple, direct method of measurement of peptic activity on routine gastric analysis samples can be performed with radioactive I^{131}-labeled albumin. This procedure is described below. It compares favorably with the established hemoglobin method of pepsin assay and it provides a clinical estimate of pepsin concentration in the gastric juice of the patient being tested.

MATERIALS AND METHODS

Gastric juice is obtained in the usual manner of gastric analysis in the fasting patient. A Levin tube is passed and the fasting residual is removed and saved. Thereafter, four 15-minute fractions of gastric juice samples are collected by constant hand suction. This series of 5 specimens constitutes the basal gastric analysis. The juice is centrifuged to spin out solid particles and mucus, and the volumes are measured.

One milliliter of radioactive iodinated albumin† of known specific activity of approximately 0.03 μc/ml. and 1 ml. of 6 per cent bovine albumin as a carrier are added to an 8 ml. test tube. The pH is adjusted to 2 by the addition of 3 ml. of Sørensen's buffer. (Sørensen's buffer: 7.51 Gm. of glycine and 5.85 Gm. of NaCl in one liter of water added to one liter of 0.1 N HCl.) One milliliter of the clear centrifuged gastric juice is then added and the mixture is incubated at approximately 37° C. for 15 minutes.

Following incubation, 1 ml. of 50 per cent trichloroacetic acid (TCA) is added. The precipitate is centrifuged and the clear supernatant is decanted into tube 1. The remaining precipitate is washed once in 5 ml. of 7 per cent TCA, spun down, and the solution decanted into tube 2. (Seven per cent TCA is used in this step since it compares with the concentration in the original sample when 50 per cent TCA has been added to it.) The fixed precipitate is dissolved in the third tube with 0.2 N NaOH and all 3 tubes brought up to the same level and counted for one minute in a Nancy Wood scintillation counter. The second tube containing the wash solution is counted 3 minutes because of its low activity. The sum of the activity of the supernatant fluids is a measure of enzymatic action on the radioactive iodinated albumin substrate. The per cent of activity is calculated by dividing the total count,

*From the Department of Medicine of the University of Kansas Medical Center, Kansas City, Kan., and the Radioisotope Section and Research Laboratory, Veterans Administration Hospital, Kansas City, Mo.

**From the Radioisotope Service and Research Laboratory, Veterans Administration Hospital, Kansas City, Mo.

†Risa, Abbott Laboratories, North Chicago, Ill.

including the count of the precipitate, into the sum of the counts of the supernatant fluids and multiplying by 100. A standard curve of reference is made by the use of 2 X crystallized pepsin* with concentrations extending over a range of 0.1 mg. through 5.0 mg. The activity of the unknown can be read directly from this (Fig. 1). The per cent of recovery is synonomous with the per cent of activity.

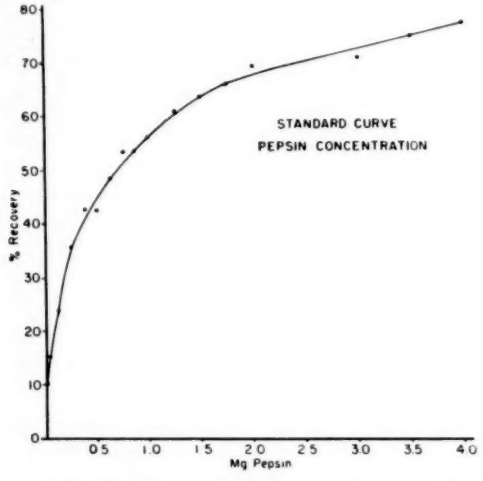

Fig. 1.—Standard curve with use of 2 X crystallized pepsin.

Since pepsin concentrations in gastric juice are usually high, the procedure can be considerably shortened without interfering with the validity of the test by performing only one step after incubation. In this short method, trichloroacetic acid is added after incubation and the supernatant fluid from centrifugation is counted. A blank of 1 ml. of iodinated albumin brought to an equal volume serves as the control and the per cent activity is calculated from this. (Table I.)

TABLE I. COMPARISON OF RESULTS OF DETAILED METHOD WITH ABBREVIATED METHOD

DETAILED METHOD OF PEPSIN ASSAY WITH RADIOACTIVE IODINATED ALBUMIN				ALTERNATIVE METHOD
SAMPLE	PER CENT ACTIVITY	MG. PEPSIN/ML. GASTRIC JUICE	PER CENT ACTIVITY	(MG. PEPSIN/ML. GASTRIC JUICE)
1	53.0	0.85	52.8	0.84
2	42.4	0.43	43.5	0.44
3	41.7	0.43	41.7	0.43
4	23.5	0.13	23.5	0.13
5	39.8	0.38	40.8	0.39
6	15.3	0.05	15.3	0.05
7	30.0	0.19	30.0	0.19
8	48.9	0.67	50.2	0.71
9	30.3	0.19	30.7	0.20
10	39.6	0.36	39.3	0.35
11	53.8	0.88	52.8	0.84
12	41.5	0.43	43.2	0.44
13	36.2	0.27	34.9	0.25
14	60.3	1.24	60.3	1.24
15	28.8	0.18	27.5	0.17

RESULTS

This procedure as described using 1 ml. of gastric juice has been used in hundreds of determinations of pepsin in gastric juice samples obtained by routine

*Nutritional Biochemical Corporation, Cleveland, Ohio.

TABLE II. BASAL GASTRIC ANALYSIS STUDY USING ONE C.C. OF GASTRIC JUICE FROM EACH 15-MINUTE SPECIMEN

TUBE NO.	SPECIMEN	VOLUME (ML.)	FREE HCL (CLINICAL UNITS)	TOTAL ACID	MEQ. PER VOLUME	TOTAL COUNTS	C.P.M. OF SUPERNATE AND WASH	PER CENT ACTIVITY	PEPSIN (MG./ML.)
1	Fasting	13	18.0	30.0	0.075	6,774	2,031	30.4	0.19
2	Basal—15 min.	8	64.0	72.0	0.186	7,270	3,480	47.9	0.61
3	30 min.	9	60.0	68.0	0.197	6,678	3,449	51.0	0.75
4	45 min.	24	32.0	42.0	0.280	6,653	3,710	55.8	0.97
5	60 min.	25	46.0	54.0	0.418	6,671	3,688	55.4	0.96

basal gastric analysis. A typical example of one such patient study is shown in Table II. Some comparative values with the hemoglobin method on "unknowns" of gastric juice are shown in Table III.

TABLE III. COMPARISON OF PEPSIN "UNKNOWNS" BY HEMOGLOBIN AND ISOTOPE METHODS

NUMBER OF "UNKNOWN"	HEMOGLOBIN METHOD	I^{131} METHOD
1	0.05 mg. pepsin	0.04 mg. pepsin
2	0.08 mg. pepsin	0.06 mg. pepsin
3	0.10 mg. pepsin	0.07 mg. pepsin
4	0.75 mg. pepsin	0.79 mg. pepsin
5	0.12 mg. pepsin	0.08 mg. pepsin

TABLE IV. INCREASING SENSITIVITY WITH DECREASE IN CONCENTRATION OF 6 PER CENT ALBUMIN CARRIER

2.5 MG. CRUDE PEPSIN WITH VARYING AMOUNTS OF 6 PER CENT ALBUMIN CARRIER	PER CENT ACTIVITY
1.0 ml.	33.7
0.9 ml.	34.4
0.8 ml.	39.8
0.6 ml.	45.9
0.5 ml.	46.9
0.3 ml.	55.2
0.1 ml.	60.2

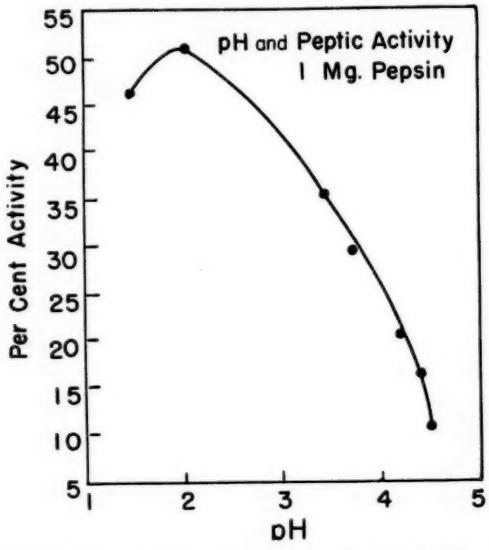

Fig. 2.—Influence of pH on peptic activity as determined by the isotope method.

DISCUSSION

Gastric juice usually has a high pepsin concentration which may not be appreciated clinically. The measured enzyme activity in milligrams of pepsin of one cubic centimeter of gastric juice determined by the method described and multiplied by volume provides an estimate of the total enzyme output in the 15-minute sample being tested. Standardization of the pepsin curve used in this method is simple and values are read directly from percentage of activity. The procedure can be applied to any routine clinical gastric analysis sample.

Since enzyme activity in gastric juice is in a concentration that usually requires dilution of the juice for the hemoglobin method, it is advantageous to perform determinations on an undiluted sample. If increased sensitivity of the test for smaller amounts of pepsin comparable to the hemoglobin method is desired, this can be achieved by decreasing the concentration of the bovine albumin carrier as shown in Table IV.

The pH of gastric juice in vivo is found to vary from time to time throughout the day in fractional samples and enzyme activity in such instances varies with this change in pH. Adjusting to a constant pH, however, reveals the potential peptic activity of the juice being sampled. Reference to peptic activity at increasing pH as shown in Fig. 2 reveals peptic activity at pH values higher than 2 and as high as 3.5 to 4. A pH range this high necessarily means the absence of free acid in the stomach, but this does not mean the complete cessation of peptic activity in the stomach. This may have some influence on the healing of peptic ulcer. It has been reported that pepsin is formed from a solution of crystalline pepsinogen after incubation at a pH as high as 4.65,[4] and according to Somers,[5] it forms from pepsinogen at a pH of 5.1. An in vivo pH of 3 or higher, therefore, does not totally eliminate peptic activity.

There seems to be no clinically effective pepsin inhibitor used in peptic ulcer therapy today. Reported suppression of activity is most probably due to an increase in pH with some concomitant decrease in peptic activity.[4] For complete inhibition of peptic activity by antacid management a constant gastric pH above 4.65 would seem to be necessary. Achievement of this high a gastric pH is not the case in the usual ulcer management employed today.

SUMMARY

A simple method for direct measurement of gastric juice peptic activity is described.

The procedure can be used in all routine gastric analysis samples and may be of clinical interest in providing an estimate of total gastric pepsin output.

Clinically significant peptic activity may be present in gastric juice in the absence of free acid.

REFERENCES

1. Osterberg, A. E., Vanzant, F. R., and Alvarez, W. C.: Studies of Gastric Pepsin. II. Secretion of Pepsin in Cases of Duodenal Ulcer and Pseudo Ulcer, J. Clin. Invest. **12**: 551-565, 1933.
2. Bucher, Gladys R., Grossman, M. I., and Ivy, A. C.: A Pepsin Method: The Role of Dilution in the Determination of Peptic Activity, Gastroenterology **5**: 501-511, 1945.
3. Polland, W. Scott, and Bloomfield, Arthur L.: A Quantitative Method for the Estimation of Pepsin, J. Clin. Invest. **7**: 45-55, 1929.
4. Hoar, Carl S., Jr., and Browning, J. R.: Plasma Pepsinogen in Peptic-Ulcer Disease and Other Gastric Disorders, A Clinical and Laboratory Evaluation, New England J. Med. **4**: 153-158, 1956.
5. Sumner, James B., and Somers, G. Fred: Chemistry and Methods of Enzymes, ed. 3, New York, 1953, Academic Press, Inc., p. 168.

THE USE OF CHICKEN ANTISERUM FOR THE RAPID DETERMINATION OF PLASMA PROTEIN COMPONENTS

II. The Assay of Human Serum Orosomucoid

Morris Goodman, Ph.D., David S. Ramsey, M.S., William L. Simpson, M.D., and Michael J. Brennan, M.D.
Detroit, Mich.

INTRODUCTION

THE rapid turbidimetric procedures developed for the measurement of human serum albumin and gamma globulin by the use of chicken antisera[1] have now been extended to the assay of a number of other human proteins. These include fibrinogen, transferrin, a beta lipoprotein, a heat labile alpha-2 glycoprotein of large molecular weight,[2] two small molecular weight alpha-2 glycoproteins,[3] and orosomucoid.[4] A major effort has been devoted to the determination of orosomucoid whose serum levels are being followed serially in a large number of cancer patients. The orosomucoid-chicken anti-orosomucoid system was found to be a somewhat different immunochemical system than those previously studied.[1] The present paper describes the optimal conditions for carrying out the orosomucoid assay and compares the serum levels of orosomucoid in normal and cancer subjects.

Orosomucoid* is a low molecular weight protein of acid isoelectric point[5-8] which resists coagulation by heat and precipitation by acids such as sulfosalicylic or perchloric but which does precipitate in phosphotungstic acid. It is a major constituent of the "seromucoid" or serum "mucoprotein"[9] fraction and exhibits a polarographic wave. Serum mucoprotein concentrations, as estimated chemically, increase in a number of pathologic states and have been extensively investigated in cancer.[5, 10-14] Orosomucoid, in contrast to the seromucoid fraction, is homogeneous both chemically[6, 7] and immunochemically[15] and probably represents a single biologic entity. Consequently, it was felt that the specific measurement of orosomucoid might provide a more meaningful index for the evaluation of clinical state than the chemical estimation of a heterogeneous fraction.[16]

MATERIAL AND METHODS

Orosomucoid was prepared from fraction (Fr) VI† of pooled human plasma by a modification of the method of Weimer and associates.[6] In a typical preparative run 10 Gm. of

From the Detroit Institute of Cancer Research and the Henry Ford Hospital, Detroit, Mich.

Supported in part by institutional grants to the Detroit Institute of Cancer Research from the American Cancer Society, Inc., the American Cancer Society, Southeastern Michigan Division, and The Kresge Foundation.

Received for publication May 8, 1957.

*Synonyms for this protein are: M-1 protein,[5] MP-1 protein,[6] and acid glycoprotein.[7]

†This fraction was generously provided to us by the Blood Products Section of the Michigan Public Health Service, Lansing, Mich.

lyophilized Fr VI were dissolved in 100 ml. of distilled water. Saturated $(NH_4)_2SO_4$ (4.1 M) was added to a concentration of 2.57 M. The precipitate was removed by centrifugation and the pH lowered to 4.9. After overnight standing at 4° C. the next precipitate was centrifuged off. The pH was lowered to 3.7 and the $(NH_4)_2SO_4$ concentration to 2.05 M. The new precipitate that formed after overnight standing at 4° C. was removed. Solid $(NH_4)_2SO_4$ was then added to the solution until full saturation was reached. The pH was maintained at 3.7. After several days standing at 4° C., the crude orosomucoid precipitate was recovered by centrifugation. The packed precipitate was dissolved by the addition of 27 ml. of distilled water. To the 45 ml. of solution that resulted, 78 ml. of sat. $(NH_4)_2SO_4$ was added. The pH was adjusted to 4.9, and the precipitate that formed after overnight standing at 4° C. was centrifuged off. The pH of the supernatant was lowered to 3.7. This time the $(NH_4)_2SO_4$ concentration was not reduced. Processing was then carried through as before. The final orosomucoid precipitate after recovery by centrifugation was dissolved, exhaustively dialyzed against distilled water, and lyophilized to yield 2 Gm. of material. The centrifugation steps were all carried out in the cold at 8,000 to 12,000 r.p.m.* The preparation had less than 1 per cent contaminants as determined by antisera to albumin and to the alpha-2 glycoproteins of Fr VI. It was also homogeneous by electrophoresis and ultracentrifugation and possessed the expected physicochemical constants.[7]† Orosomucoid preparations of less purity could be detected during the recycling by the greenish color of the pH 3.7 supernatants. Cautious fractional addition of sat. $(NH_4)_2SO_4$ then sufficed to salt out the contaminants.

During the current studies, 31 healthy hens, 6 to 12 months of age and of various breeds, underwent immunization. Antibody responses were quite variable by each of the injection schedules followed. The highest percentage of usable sera were produced by a schedule in which each bird received intramuscularly into 4 spots an injection of 200 mg. of orosomucoid well mixed with aluminum hydroxide gel. Ten days later the blood was collected and processed according to the procedure of Silberberg and associates.[15] About 30 per cent of the birds yielded negative serum. Efforts are continuing to find more effective injection procedures.‡

The specificity of each antiserum was evaluated by carrying out flocculation reactions and Oudin tests[17] with Fr VI and its various components: orosomucoid, albumin, and two purified alpha-2 glycoprotein preparations.§ A number of the Oudin tests were carried out in 13 per cent NaCl instead of 8 per cent. Antisera reacted at 13 per cent NaCl were first incubated for 2 hours at 38° C. after addition of the proper amount of NaCl. The residual fibrinogen, having flocculated, was then removed by centrifugation. Antisera that were completely specific for orosomucoid yielded only one band in the Oudin tests and only precipitated with Fr VI and orosomucoid. Potency was estimated in a qualitative manner by noting the degree of flocculation when 1, 10, and 100 mcg. of orosomucoid in 0.1 ml. aliquots were mixed, respectively, with 0.2 ml. portions of the antiserum dilution (1 to 2 after processing). Antisera showing comparable potencies and specificities were pooled together.

Biuret determinations, employing the reagent of Cornall and associates,[18] were carried out as previously described.[1] Hexose and seromucoid determinations were performed according to methods outlined by Winzler.[4]

RESULTS

Effect of NaCl Concentration.—The effects of NaCl concentration on avian precipitin systems have been intensively investigated.[19-21] Specific precipitation was found to increase on raising the NaCl concentration from 1 to 8 per

*A Serval or high speed head No. 855 of the International proved convenient for this purpose.

†We are indebted to Dr. Karl Schmid for performing the electrophoretic and ultracentrifuge analyzes of this preparation.

‡Procedures employing combined intermuscular (with adjuvant) and intravascular injections appear to hold promise.

§These were supplied to us by Dr. Karl Schmid.

cent. With antialbumin and antigamma globulin systems further elevations above 8 per cent caused a broadening of the zone of maximal precipitation into the far antigen excess zone but did not markedly increase the precipitation at equivalence. The difference in precipitation on varying salt concentration was dependent on the concentration of reactants—the higher the salinity the smaller the inhibition of precipitation on lowering the concentration of reactants.[19, 20]

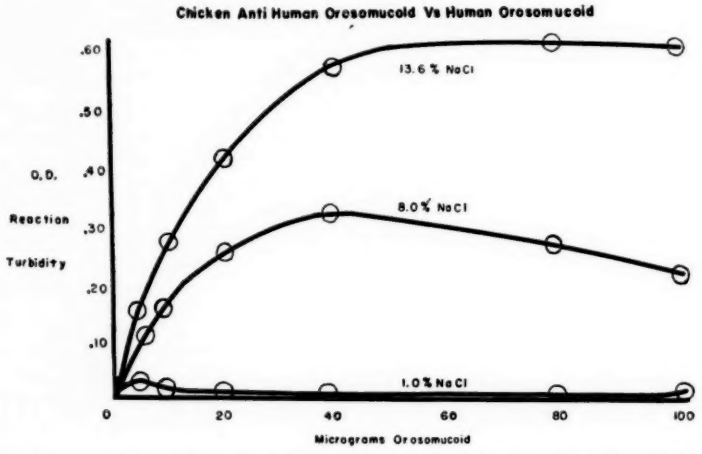

Fig. 1.—Effect of NaCl concentration on precipitate formation-chicken antiorosomucoid serum (C-17) plus orosomucoid; 0.1 ml. antigen + 0.4 ml. ¼ C-17. One hour incubation at 38° C. Reaction turbidities recorded in Beckman spectrophotometer set at 450 mμ.

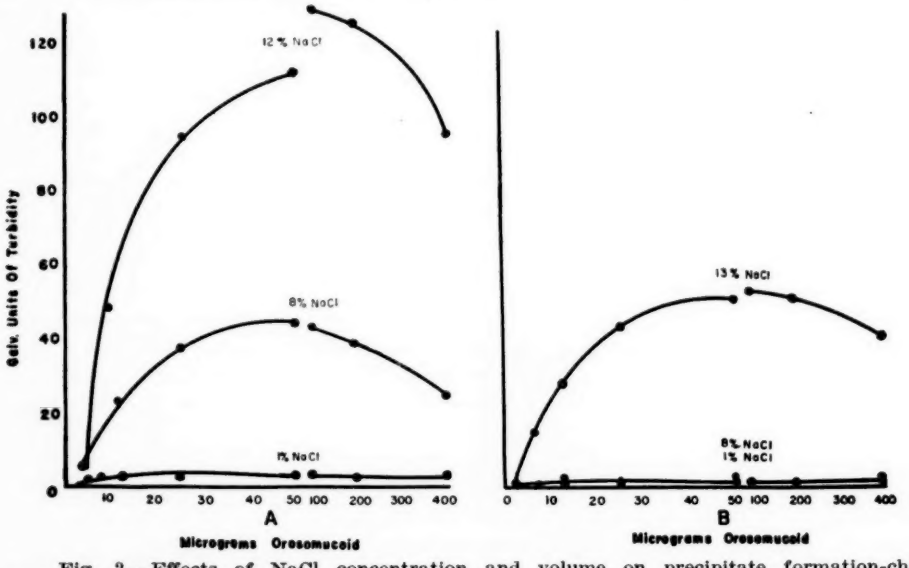

Fig. 2.—Effects of NaCl concentration and volume on precipitate formation-chicken antiorosomucoid serum (P-17) plus orosomucoid. *A*, 5.5 ml. reaction volume containing 0.5 ml. P-17; 20-minute incubation at 38° C. *B*, 5.5 ml. reaction volume containing 0.25 ml. P-17; 45-minute incubation at 38° C. Nephelometric determination of turbidity.

It occurred to us that the chicken antiorosomucoid-orosomucoid system might show these salt effects to a more pronounced degree and that it might be advantageous to react this system at 12 to 15 per cent NaCl instead of 8 per

cent. Figs. 1 and 2 demonstrate this to be the case. Precipitation failed to develop in 1 per cent NaCl and was much less at 8 per cent than at 12 or 13.6 per cent NaCl. This was true in the antibody excess zone as well as the equivalence and antigen excess zones.

At the high dilution of reactants employed in the nephelometric method the salt effects were accentuated. After the initial incubation at 38° C. the precipitin reactions in 12 to 13 per cent NaCl were essentially complete except in the far antibody excess zone. There increased turbidity developed after overnight standing. The slower rate of precipitation at small concentrations of orosomucoid was more marked in 8 per cent NaCl than in 12 to 13 per cent and extended to larger concentrations of the antigen. Indeed, with half the quantity of antiserum (see Fig. 2, *B*) detectable precipitation in 8 per cent NaCl only developed after overnight standing. In 1 per cent NaCl precipitation was insignificant even after 5 days incubation at 5° C.

Procedures for the Assay of Orosomucoid.—Based on the above results the following orosomucoid assay method was set up with an antiserum (P-17)* exhibiting high potency and specificity. A 0.5 ml. of the antigen solution at 13 per cent NaCl was pipetted into a test tube. Then 0.5 ml. of a 3:5 dilution of P-17, adjusted to 13 per cent NaCl, was introduced. After 15 minutes incubation at 38° C., 4.7 ml. of 14 per cent NaCl was added. These two additions were effected in each case with an appropriate automatic Cornwall syringe to permit rapid mixing of the reagents. The contents of the tube were transferred to a calibrated cuvette, and turbidity was read in the Coleman model 7 photonephelometer. The standards varied from 0 mcg. orosomucoid to 25 mcg., the 0 tube providing the reagent blank upon which the instrument was set at zero galvanometer units. A 0.5 ml. of 1:100 dilution of human serum in 13 per cent NaCl was chosen for sample assay. This amount permitted ready quantitation of the elevated orosomucoid values occurring in certain pathologic states, normal values falling on the lower quarter of the calibration curve. The galvanometer units of turbidity of typical standard tube reactions are presented in column B of Table I.

An orosomucoid assay which required only one-half to one-third the amount of antiserum used in the nephelometric procedure was also worked out with the Beckman D.U. spectrophotometer.† The reactions were developed in 0.5 ml. volume at 13 per cent NaCl in 10 by 75 mm. test tubes. After incubation at 38° C. for 1 hour, by which time flocculation was complete, each reaction tube was shaken and its contents transferred to a microcell requiring a minimum volume of 0.35 ml. The turbidity as optical density units (O.D.) was read against a reagent blank consisting of antiserum plus 13 per cent NaCl placed in a companion microcell. The readings were taken at 340 mμ with the ultraviolet source of light. Under these spectrometric conditions the largest turbidity readings could be obtained with the least interference from the protein and hemoglobin contents of the reaction mixtures. Standards were varied

*A pool of 4 antisera of similar characteristics, each at the routine 1:2 dilution in 8 per cent NaCl.

†With other sensitive laboratory spectrophotometers, such as the Bausch and Lomb Spectronic 20 and the Universal Coleman, comparable assay procedures can be worked out with suitable microcells.

from 0 to 12.5 mcg. orosomucoid, and the readings at the top portion of the calibration curve were 0.400 to 0.500 O.D. units. The routine quantity of human serum chosen for assay was 0.002 ml. (either 0.1 ml. of a 1:50 dilution or 0.2 ml. of a 1:100 dilution in 13 per cent NaCl).

TABLE I. EFFECT OF VOLUME AND TIME ON THE STANDARD TUBE REACTIONS EMPLOYED IN THE NEPHELOMETRIC METHOD

	1 ML. INITIAL REACTION VOLUME						5.7 ML. INITIAL REACTION VOLUME
	A		B		C		D
OROSOMUCOID (MCG.)	7.5″ 38° C. + 45″ 25° C.		15″ 38° C. + 45″ 25° C.		30″ 38° C. + 45″ 25° C.		60″ 38° C.
0	0	0	0	0	0	0	0
3.13	4.5	9	13	13	14	13	3
6.25			21	21.5			17
12.5			40	39			36
25.0	61.5	62	63	63	63.5	62.5	54

A = 0.5 ml. orosomucoid (at 13 per cent NaCl) + 0.5 ml. 3:5 P-17 (at 13 per cent NaCl); incubate 7.5 min. 38° C., then add 4.7 ml. 14 per cent NaCl.

B = 0.5 ml. orosomucoid (at 13 per cent NaCl) + 0.5 ml. 3:5 P-17 (at 13 per cent NaCl); incubate 15 min. 38° C., then add 4.7 ml. 14 per cent NaCl.

C = 0.5 ml. orosomucoid (at 13 per cent NaCl) + 0.5ml. 3:5 P-17 (at 13 per cent NaCl); incubate 30 min. 38° C., then add 4.7 ml. 14 per cent NaCl.

D = 0.5 ml. orosomucoid (at 13 per cent NaCl) + 4.7 ml. 14 per cent NaCl + 0.5 ml. 3:5 P-17 (at 13 per cent NaCl).

During the past two years over 1,500 human sera have been assayed for orosomucoid. The above turbidimetric procedures have been employed in the assay of the last 800 samples. To help insure uniformity on going from one antiserum to the next, the orosomucoid value of a large pool of human serum from patients with elevated values was calculated by repeat analyzes with our best reagents, a highly potent and specific antiorosomucoid serum (P-17) and a highly pure orosomucoid preparation (oroso 8). The calibrated serum pool (PCS) was divided into small vials and stored in a deep-freeze. As needed, portions have been thawed to provide standards for the orosomucoid assay.

The calibration curves obtained by reacting P-17 with PCS and oroso 8 were very similar, but not identical. As constructed from the orosomucoid value (250 mg. per cent) assigned to PCS, the curves intersected each other at their mid-portions. The divergence of the 2 curves was found in some cases to be more pronounced with other orosomucoid preparations and on reacting different antisera. These data are illustrated in part by Fig. 3. It can be seen that oroso 16 afforded less precipitation on a comparable weight basis than oroso 8. Oroso 16 was prepared by extensive refractionation of a heterogeneous preparation showing a lower salting out range in $(NH_4)_2SO_4$ solutions than oroso 8. The only contaminant detected immunochemically in oroso 16 was a trace of alpha-2 glycoprotein.

Table II demonstrates that with PCS as the standard the same sample assay values within experimental error were obtained by the various antiorosomucoid sera employed. Some of these sera (e.g., P-30) lacked complete specificity and others (e.g., C-124) exhibited inhibition of precipitation in the far antibody excess zone. The human serum samples could be stored for as long as

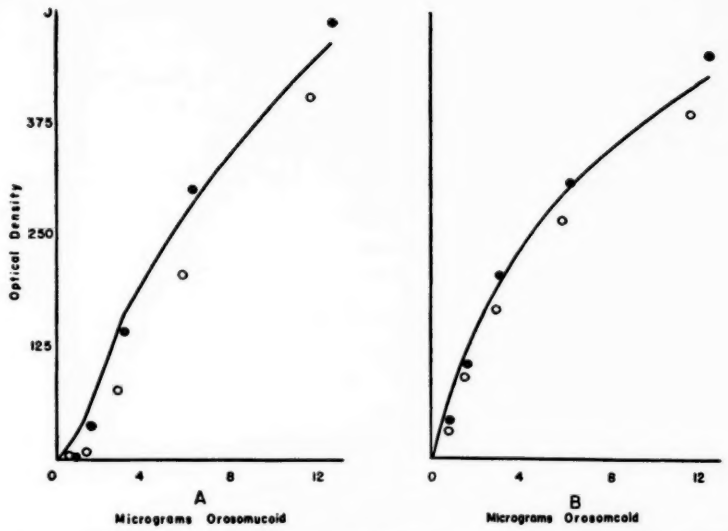

Fig. 3.—Comparison of the PCS calibration curve of antiorosomucoid sera with antibody excess zone reactions of two orosomucoid preparations. Solid line = PCS calibration curve. Solid circle = oroso 8 reactions. Open circle = oroso 16 reactions. *A*, 0.2 ml. Ag + 0.3 ml. Ab (1:2.52 C-124); 13 per cent NaCl; 1 hour incubation at 38° C. *B*, 0.2 ml. Ag + 0.3 ml. Ab (1:2 P-17); 13 per cent NaCl; 1 hour incubation at 38° C. Reaction turbidities recorded in Beckman spectrophotometer set at 340 mμ.

TABLE II. IDENTITY OF SAMPLE ASSAY VALUES BY DIFFERENT ANTIOROSOMUCOID SERA

SAMPLE NO.	C-17* (MG. %)	P-17 (MG. %)	P-18 (MG. %)	P-30† (MG. %)	C-124‡ (MG. %)
532	120				115
621	202				203
644	140				139
682	235				227
790		70			80
853		263	243		
856		193	181		
858 (N)§		57	50		
921		125			125
935		72			101
1,074 (N)		66		70	
1,076		235	250		227
1,079		238	225		255
1,082 (N)		78		80	
1,106		83			80
1,200 (N)		96	88		93
1,202 (N)		67		70	68
1,203 (N)		75		60	
1,212 (N)			70		56
1,239			210		200
1,249			325		350
1,252			80		85
1,301				145	151
1,313				105	105
1,334				176	160
1,383				345	353
1,423				200	235
1,441				215	212
1,480				220	195

*The current turbidimetric method was being evolved at the time that C-17 was employed. The standard, then, was made up from an individual calibrated cancer serum of high orosomucoid content. It served as the forerunner for PCS.

†P-30 yielded a weak reaction with an alpha-2 glycoprotein in addition to its strong reaction for orosomucoid.

‡The determinations with C-124, our most recent antiserum, were carried out almost a year after C-17 had been consumed in testing.

§(N) designates a sample from a normal donor.

10 months in the liquid state at -2° C. without apparent effect on the orosomucoid assay. The duplication error of the method was ± 9 per cent in the hands of laboratory technicians.

The reliability of the orosomucoid assay was further investigated by adding known quantities of purified orosomucoid to solutions of other serum proteins. Tables III and IV demonstrate that the nonorosomucoid components of such mixtures in no way affected the immunochemical assay. On the other hand, by chemical estimation only 50 to 60 per cent of the added orosomucoid could be recovered due to its coprecipitation in perchloric acid with the albumin and gamma globulin.

TABLE III. EFFECT OF HUMAN SERUM ON DETERMINATION OF KNOWN QUANTITIES OF OROSOMUCOID

A SAMPLE CONTENT*	B KNOWN OROSO. CONTENT (MG./ML.)	C OROSO. CONTENT BY IMMUNOCHEMICAL ASSAY† (MG./ML.)
1. 1 ml. saline + 0.1 ml. 2.75 mg. oroso 7	2.50‡	2.56
2. 1 ml. 1 mg. oroso 8 + 0.1 ml. saline	0.91‡	0.80
3. 1 ml. 1 mg. oroso 8 + 0.1 ml. 2.75 mg. oroso 7	3.41‡	3.30
4. 1 ml. Z-948 + 0.1 ml. saline		0.74
5. 1 ml. Z-948 + 0.1 ml. 2.75 mg. oroso 7	3.24§	2.94
6. 1 ml. Z-901 + 0.1 ml. saline		1.25
7. 1 ml. Z-901 + 0.1 ml. 2.75 mg. oroso 7	3.75‖	3.78

*Mixtures of two purified orosomucoid preparations either with saline or with human serum. Z-948 and Z-901 are human serum samples.

†The nephelometric procedure was employed.

‡From biuret determinations of the two purified orosomucoid preparations. The biuret value of orosomucoid, employing human serum albumin as the biuret standard, was multiplied by the factor 1.47 to obtain the total weight of orosomucoid.

§The sum of row 1 (column B) + row 4 (column C).

‖The sum of row 1 (column B) + row 6 (column C).

TABLE IV. THE EFFECT OF ALBUMIN AND GAMMA GLOBULIN CONCENTRATION OF THE RECOVERY OF OROSOMUCOID AS ASSAYED BY THE SEROMUCOID TEST AND THE IMMUNOCHEMICAL TEST

SAMPLE CONTENT	MG. OROSOMUCOID PER ML. AS ASSAYED BY: SEROMUCOID TEST*	IMMUNOCHEMICAL TEST†
A. 1 mg. human orosomucoid 7 per ml. 50 mg. Rwk Fr V (human) per ml. 25 mg. Fr II (human per ml. Buffered 0.9 per cent NaCl, pH 7.6	0.53	1.03
B. 1 mg. human orosomucoid 7 per ml. 25 mg. Rwk Fr V (human) per ml. 25 mg. Fr II (human) per ml. Buffered 0.9 per cent NaCl, pH 7.6	0.60	0.97
C. 1 mg. human orosomucoid 7 per ml. Buffered 0.9 per cent NaCl, pH 7.6	1.00	0.95

*Seromucoid hexose assay as described by Winzler[4] employing sample C as the standard for A and B.

†The Beckman spectrophotometric procedure was employed.

Serum Orosomucoid Levels Compared to Mucoprotein Levels.—Fig. 4 compares the serum orosomucoid levels of 74 nomal adults and 142 cancer patients.* The normal samples were drawn from healthy laboratory workers and

*Since the results were tabulated, a considerable number of additionai patients have been added to the series.

from hospital blood donors. The cancer samples were from patients with various malignancies, mainly in advanced inoperable stages. Serial determinations have been performed on a number of these patients for months at a time, but for purposes of this paper only one value per patient is recorded. In each

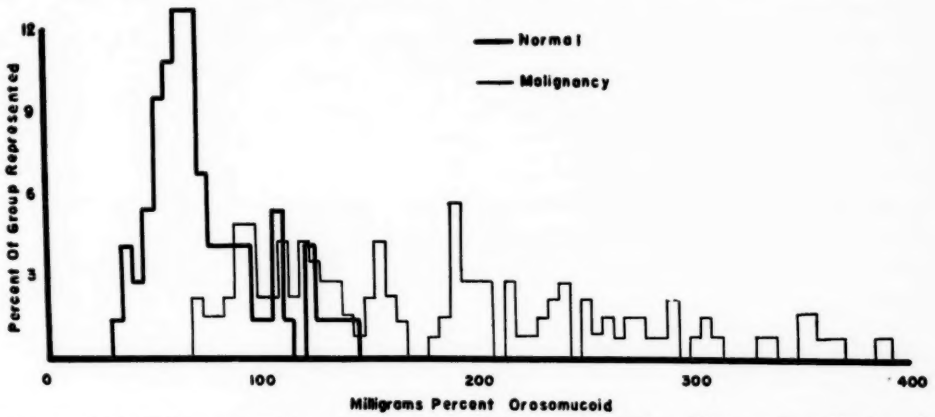

Fig. 4.—Distribution of serum orosomucoid levels in normal adults and patients with malignancy: 74 normals compared to 142 patients.

case the value was the first one determined on the patient during the time span encompassed by receipt of samples Z-715 to Z-1500. The orosomucoid values in the normals ranged from 37 mg. per cent to 145 mg. per cent with a mean of 76 mg. per cent and in the cancer group ranged from 68 mg. per cent to 390 mg. per cent with a mean of 177 mg. per cent. In spite of the wide range of values in the cancer sera, there was a striking correspondence between the increased mean of our series and those reported by various investigators for serum mucoprotein concentration.[4, 10, 11, 14] This is to be expected since orosomucoid is the principal component responsible for the chemical estimations.

TABLE V. COMPARISON OF MUCOPROTEIN CONTENT IN NORMAL AND CANCER SERUM BY VARIOUS ASSAY METHODS

	NORMAL	CANCER	RATIO $\frac{C}{N}$	REFERENCE
Immunologic orosomucoid test*	76.0 ± 2.99†	177.0 ± 6.49†	2.33	Present experiments
Polarographic filtrate test‡	1.0	2.4 ± 0.03	2.40	Brdicka et al.[11]
Mucoprotein§	2.7 ± 0.05	6.1 ± 0.13	2.26	Winzler and Smyth[10]
Mucoprotein‖	136.5 ± 3.93	308.0 ± 15.4	2.25	Lockey et al.[14]

*Milligram per cent orosomucoid.

†Standard error.

‡Recorded as ratio $\frac{\text{mm. wave length for cancer serum}}{\text{mm. wave length for normal serum}}$.

§Milligram per cent of tyrosine of the serum fraction soluble in perchloric acid but insoluble in phosphotungstic acid.

‖Milligram per cent of mucoprotein, that serum fraction soluble in sulfosalicyclic acid but insoluble in phosphotungstic acid.

A comparison of values obtained by these different tests is presented in Table V. Nine tuberculosis serum samples and 12 pregnancy samples were also analyzed for orosomucoid content. There were elevated values in the tuberculosis group, but in the pregnancy group orosmucoid was at normal concentration. This, too, is in accord with the results of chemical serum mucoprotein

estimation. It is well to note that the range and nonspecificity in serum levels of orosomucoid and the chemical mucoprotein fractions are so great as to preclude their being of diagnostic value in cancer.

DISCUSSION

The current study represents an extension of our efforts to develop an inclusive system for the quantitation of human plasma proteins by the use of chicken antisera. Pertinent to this objective has been the observation that at 13 per cent NaCl far more potent reactions can be obtained with antiorosomucoid serum than at 8 per cent NaCl. To varying degrees this high salt effect is manifested by all the chicken antiserum-antigen systems studied so far. The weaker or more dilute an antiserum the greater is the difference in precipitation between 13 and 8 per cent. Consequently, antisera in small supply such as we have produced to several alpha-2 glycoproteins are now being stored and reacted at the higher salinity.

The current work further substantiates our previous observation[1] that certain antisera lacking complete specificity can be used to gather reliable data. For antiserums such as P-30 this may have been fortuitous in that the "unwanted" antibodies were directed to a protein whose human serum concentration appears to be very low.* However, our use of a calibrated pool of human serum as the assay standard may well have neutralized slight differences in reactivity between the various antiorosomucoid sera.

The mean serum concentration of orosomucoid in normals was found to be 76 mg. per cent. This value is lower than the 103 mg. per cent reported by Silberberg and associates[15] and the 119 mg. per cent reported in our preliminary studies.[22] The orosomucoid preparations used as the assay standards in the earlier work may have been less pure or more altered in specificity than the orosomucoid used to calibrate PCS, the routine standard of the current study. Our value of 76 mg. per cent is in accord with the data gathered for the seromucoid or serum mucoprotein fraction. De La Huerga and associates[23] have established by a turbidimetric assay that the mean value for the chemical fraction in normal serum is 79.4 mg. per cent. This value corresponds to mean values for other groups of normals obtained by biuret,[24] tryrosine,[10] and hexose[4, 10] methods.† Upon adjusting for the estimated 30 per cent loss during perchloric acid precipitation,[9] the total normal serum mucoprotein concentration can be considered 113 mg. per cent, 67 per cent of which is orosomucoid. These results agree with the observed electrophoretic heterogeniety of seromucoid[16] in which its major component (MP-1) possesses the physicochemical properties of orosomucoid.

The specific immunochemical measurement of orosomucoid has greater accuracy than the various serum mucoprotein assay methods. These latter have an inherent technical error in that the recovery of seromucoid is dependent on the concentration and type of proteins in the serum sample under assay.

*This protein is concentrated in the Zn precipitable alpha-2 glycoprotein fraction characterized by Schmid.[3]

†De La Huerga and associates[23] discuss the correspondence of these various chemical methods of assaying the serum mucoprotein fraction.

Breakdown products of proteins can also occur in the chemical fraction after storage of serum. Furthermore, the relative distribution of the components in seromucoid may vary according to the physiologic stimuli. Mandel and associates[25] have pointed out the uncertainty this introduces into the evaluation of seromucoid determinations. In diseases such as malignancy and tuberculosis, both orosomucoid and seromucoid increase in concentration over normal levels and show a comparable spread of values in randomly selected patients. Yet the two measures may not necessarily parallel each other in individual patients subjected to various forms of treatment. For example Bollet[26] has reported that in some patients cortisone administration caused an increase in the total protein of seromucoid and a concomitant relative decrease in the hexosamine of the fraction. Our own work[27] indicates that in cancer the orosomucoid level is not directly affected per se by cortisone or a number of other therapeutic compounds, but is affected by a gross change in the clinical state of the patient, increasing with clinical deterioration and decreasing with clinical improvement. That seromucoid and orosomucoid levels need not be closely correlated is also indirectly suggested on comparison of these measures to the presence or absence in human sera of C-Reactive Protein (CRP). Shetlar and associates[28] have claimed a close correlation between the elevation of seromucoid concentration and the appearance of CRP. On the other hand our data (unpublished) has shown a number of samples in which orosomucoid concentration was elevated and CRP absent. The reverse was also found. The possible differences in data gathered by the seromucoid and the immunochemical orosomucoid assay methods point to the need to analyze the constituents of serum in terms of discrete protein species. The fluctuations in serum concentration of such proteins as orosomucoid may provide a more definitive characterization of the processes operative in disease.

SUMMARY

The measurement of serum orosomucoid concentration by specific chicken antiorosomucoid serum has been demonstrated to be a practical laboratory method. Rapid turbidimetric procedures for assay were worked out in which the reactions were carried out in 13 per cent NaCl. The choice of this high salinity resulted from the observation that the effects of NaCl concentration were more pronounced in the chicken antiorosomucoid system than in other chicken antiserum systems. The average serum orosomucoid level of normal adults was found to be 76 mg. per cent ± 2.99 (s.e.). Marked increases of serum orosomucoid concentration were often found in malignancy, and there was also a wider range of levels than in normal subjects. The relationship of the orosomucoid determination to various serum mucoprotein assay methods was discussed.

REFERENCES

1. Goodman, M., Ramsey, D. S., Simpson, W. L., Remp, D. G., Basinski, D. H., and Brennan, M. J.: The Use of Chicken Antiserum for the Rapid Determination of Plasma Protein Components. I. The Assay of Human Serum Albumin and Gamma Globulin, J. Lab. & Clin. Med. **49**: 151, 1957 .
2. Brown, R. K., Baker, W. H., Peterkofsky, A., and Kauffman, D. L.: Crystallization and Properties of a Glycoprotein Isolated from Human Plasma, J. Am. Chem. Soc. **76**: 4244, 1954.

3. Schmid, K.: Isolation and Characterization of Glycoproteins from Human Plasma, J. Am. Chem. Soc. **75**: 2532, 1953.
4. Winzler, R. J.: Determination of Serum Glycoproteins; Methods of Biochemical Analysis, vol. II, New York, 1955, Interscience Publishers, Inc.
5. Petermann, M. L., and Hogness, K. R.: Electrophoretic Studies on the Plasma Proteins of Patients With Neoplastic Disease. II. An Acid Protein Present in the Plasma, Cancer **2**: 104, 1948.
6. Weimer, H. E., Mehl, J. W., and Winzler, R. J.: Studies on the Mucoproteins of Human Plasma. V. Isolation and Characterization of a Homogeneous Mucoprotein, J. Biol. Chem. **185**: 561, 1950.
7. Schmid, K.: Preparation and Properties of Serum and Plasma Proteins. XXIX. Separation from Human Plasma of Polysaccharides, Peptides and Proteins of Low Molecular Weight. Crystallization of an Acid Glycoprotein, J. Am. Chem. Soc. **75**: 60, 1953.
8. Smith, E. L., Brown, D. M., Weimer, H. W., and Winzler, R. J.: Sedimentation, Diffusion, and Molecular Weight of a Mucoprotein from Human Plasma, J. Biol. Chem. **185**: 569, 1950.
9. Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M.: Studies on the Mucoproteins of Human Plasma. I. Determination and Isolation, J. Clin. Invest. **27**: 609, 1948.
10. Winzler, R. J., and Smyth, I. M.: Studies on the Mucoproteins of Human Plasma. II. Plasma Mucoprotein Levels in Cancer Patients, J. Clin. Invest. **27**: 617, 1948.
11. Brdicka, R., Novak, F. V., and Klumpor, J.: Critical Examination of the Polarographic Test for Cancer in Deproteinated Sera. Acta radiol. et cancerol. bohem. et morav. **2**: 27, 1939.
12. Robinson, A. M.: Polarographic Analysis of Normal and Pathological Sera, Brit. J. Cancer **2**: 360, 1948.
13. Butler, L. O.: The Polarographic Serum Test and Other Serum Tests in the Prognosis of Cancer, Brit. J. Cancer **5**: 225, 1951.
14. Lockey, E., Anderson, A. J., and Maclagan, N. F.: Urine and Serum Mucoproteins in Cancer and Other Diseases, Brit. J. Cancer **10**: 209, 1956.
15. Silberberg, S., Goodman, M., Kefalides, N. A., and Winzler, R. J.: Immunochemical Determination of Orosomucoid, Proc. Soc. Exper. Biol. & Med. **90**: 641, 1955.
16. Mehl, J. W., Humphrey, J., and Winzler, R. J.: Mucoproteins of Human Plasma. III. Electrophoretic Studies of Mucoproteins from Perchloric Acid Filtrates of Plasma, Proc. Soc. Exper. Biol. & Med. **72**: 106, 1949.
17. Oudin, J.: Methode d'analyse immunochimique par precipitation specifique en milieu gelifie, Compt. rend. Acad. sc. **222**: 115, 1946.
18. Cornall, A. G., Bardawill, C. J., and David, M. M.: Determination of Serum Proteins by Means of the Biuret Reaction, J. Biol. Chem. **177**: 751, 1949.
19. Goodman, M., Wolfe, H. R., and Norton, S.: Precipitin Production in Chickens. VI. The Effect of Varying Concentrations of NaCl on Precipitate Formation, J. Immunol. **66**: 225, 1951.
20. Goodman, M., and Wolfe, H. R.: Precipitin Production in Chickens. VIII. A Comparison of the Effect of Salt Concentration on Precipitate Formation of Pheasant, Owl, and Chicken Antisera, J. Immunol. **69**: 423, 1952.
21. Goodman, M., Wolfe, H. R., and Goldberg, R.: Precipitin Production in Chickens. XII. The Effects of Variation in Ionic Species and Concentration on Precipitate Formation, J. Immunol. **72**: 440, 1954.
22. Brennan, M. J., Goodman, M., and Simpson, W. L.: Orosomucoid, C-Reactive Protein and Albumin Variances Measured Immunochemically in Cancer Patients. II. Clinical Correlations, Proc. Am. A. Cancer Res. **2**: 96, 1956.
23. De La Huerga, J., Dubin, A., Kushner, D. S., Dyniewicz, H. A., and Popper, H.: Studies of Serum Mucoprotein (Seromucoid). I. A Turbidimetric Method, J. Lab. & Clin. Med. **47**: 403, 1956.
24. Greenspan, E. M., and Dreiling, D. A.: Serum Mucoprotein Level in Differentiation of Hepatogenic From Obstructive Jaundice, A. M. A. Arch. Int. Med. **91**: 474, 1953.
25. Mandel, E. E., Gorsuch, T. L., and Cooper, G. E.: Seromucoid in Hepatobiliary Disease, Clin. Chem. **1**: 221, 1955.
26. Bollet, A. J.: The Hexosamine Content of the Serum Globulins in Normal and Pathological Sera, J. Clin. Invest. **36**: 51, 1957.
27. Simpson, W. L., Goodman, M., and Brennan, M. J.: Consideration of Selected Systemic Reactions to Cancer as Objective Indices of Response to Therapy, Proc. Am. A. Cancer Res. **2**: 250, 1957.
28. Shetlar, M. R., Bullock, J. A., Shetlar, C. L., and Payne, R. W.: Comparison of Serum C-Reactive Protein, Glycoprotein, and Seromucoid in Cancer, Arthritis, Tuberculosis and Pregnancy, Proc. Soc. Exper. Biol. & Med. **88**: 107, 1955.

THE CHEMICAL ESTIMATION OF EPINEPHRINE AND NOREPINEPHRINE IN HUMAN AND CANINE PLASMA

II. A Critique of the Trihydroxyindole Method.

Henry L. Price, M.D.,* and Mary L. Price, A.B.
Philadelphia, Pa.

INTRODUCTION

THE ethylenediamine condensation method of Weil-Malherbe and Bone[1] and its modifications have been extensively used to estimate the concentrations of epinephrine (E) and norepinephrine (N) in plasma, blood, tissues, and urine. Recently, it has been reported that this method is not sufficiently specific for these purposes.[2, 3]

The trihydroxyindole method of Lund[4] is more specific, but has been found to lack sufficient sensitivity to detect the extremely low concentrations of E and N which normally occur in human and canine plasma.[4-6]

In the work to be reported, means of increasing both the sensitivity and specificity of the trihydroxyindole method have been sought. It has been found: (1) that the quantities of E and N present in a mixture can be estimated fluorometrically rather than by differential oxidation,[4] thereby reducing the volume of plasma needed for analysis by one-third; (2) that the fluorescence produced by E and N is increased while that of certain interfering substances is reduced by adjusting the pH of the reaction products; and (3) that the precision of the determinations can be improved by a new method of preparing blanks. With these modifications 0.001 μg E or 0.002 to 0.003 μg N can be detected in plasma eluates. Satisfactory recovery of these quantities of E and N is demonstrated below, and the concentrations of these substances occurring in normal human plasma are estimated.

METHODS

Blood samples are collected in syringes moistened with heparin and are iced immediately. The plasma is separated as soon as possible by centrifuging the blood at 750 × g for 20 minutes. Chromatography of the plasma is completed within 3 hours after the blood is drawn.

A. *Alumina Column Chromatography.*—The columns used consist of glass tubes 25 cm. in length with an upper portion 5 cm. long and 0.8 cm. in diameter, a bulbous central part of 30 ml. capacity, and a lower portion 0.6 cm. in inside diameter and 10 cm. long. The bottom of the tube is crimped slightly to hold a glass wool plug which supports the alumina. With

From the Department of Anesthesiology, Hospital of the University of Pennsylvania and the Harrison Department of Surgical Research, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

This study was supported in part by grant H-1568 from the U. S. Public Health Service.
Received for publication May 10, 1957.

*Wellcome Associate Professor of Anesthesiology, School of Medicine, University of Pennsylvania.

the column half-filled with distilled water and the glass wool plug in position, 1.0 Gm. of alumina* is poured into the top of the column and allowed to settle. Trapped air is removed from the alumina by agitation with a glass rod introduced through the top of the column before settling is complete. The supernatant water is forced through the column, using air pressure, until its level is just above that of the alumina. The alumina is washed with 5 ml. of glass-distilled water, and is then ready for use.

The plasma pH is adjusted to 8.0 ± 0.3 by swirling it in a beaker, causing loss of carbon dioxide and increase in pH, after which it is poured onto the alumina column. The plasma is forced through at an even rate (1 to 2 ml./min.) by suitable adjustments of air pressure. When the plasma level has reached that of the top of the alumina, 5 ml. 0.2 N sodium acetate followed by 5 ml. glass-distilled water is forced through the column. Columns which have been allowed to run dry at any point before elution are discarded. The alumina is eluted with 6 ml. of 0.3 N acetic acid. Eluates are collected in glass-stoppered tubes, in which they may be frozen and stored for several weeks before analysis, without significant loss of E or N.

B. *Analysis of Eluates.*—Merck reagent grade chemicals are used throughout, except as noted. The pH of each eluate fraction (2.0 to 3.0 ml.) to be analyzed is adjusted to 6 by drop-wise addition of 1 N sodium hydroxide after the addition of 0.04 per cent bromthymol blue indicator (Harleco), and 1.0 ml. 1 N sodium acetate buffer (pH 7.6 to 8.0). The correct end point is indicated by the appearance of a persistent light-green color. Samples which become more alkaline, as indicated by the appearance of a persistent blue color, should be discarded.

An eluate fraction is then run as a *sample* by adding 0.1 ml. of 0.25 per cent potassium ferricyanide solution, mixing, and allowing the mixture to stand 5 minutes. After this 0.10 ml. of 2.0 per cent ascorbic acid is added, followed immediately by 0.9 ml. of 20 per cent sodium hydroxide. The mixture can then be diluted to standard volume (6 to 7 ml.) and read in a fluorometer, but for reasons to be discussed later, we routinely convert the pH of the mixture to approximately 5 by the addition of 2.0 ml. of 5 N acetic acid 4 minutes after the addition of ascorbic acid and before diluting to final volume.

A *blank* is prepared from another equal fraction of the eluate after titrating it to pH 6. Ascorbic acid, followed by ferricyanide and sodium hydroxide are added in the same amounts used to prepare samples. Four minutes later the pH is brought to 5 by adding 5 N acetic acid as in the samples.

Samples and blanks are read in a Farrand photofluorometer at equal times (7 and 10 minutes) after the addition of sodium hydroxide. Light of two wave lengths (391 to 408 and 436 mμ) is used to excite the solution; the emission is read at 500 mμ. The filter combinations used are: (exciting) 400 mμ Farrand interference plus Wratten No. 35, and 436 mμ Farrand interference plus Wratten No. 35; and (emitting) 500 mμ Farrand interference plus Wratten No. 57.

Standard additions of E and N (Sterling-Winthrop: 0.01 to 0.05 μg of base) are made to plasma eluates or to "blank" eluates prepared by running reagents only through alumina columns and eluting with acetic acid as described above. Additions made to reagents which have not been exposed to alumina are unacceptable for analytic purposes because the fluorescence observed per microgram of E and N and the ratios of fluorescence observed using the 400 and 436 mμ primary filters are modified by the presence of the acetic acid eluate of alumina.

Calculations of E and N concentration are performed by comparing the fluorescence obtained per microgram of added E and N with the net fluorescence (sample reading minus blank reading) observed in the unknown. This procedure yields simultaneous equations which can be solved algebraically:

Equation 1: $F\ 400 = mE + nN$

Equation 2: $F\ 436 = oE + pN$, where F_{400} and F_{436} are the net (sample minus blank) number of galvanometer divisions observed when the sample is excited by light of the two

*Woelm, nonalkaline, Grade I, Alupharm Chemical Co., New Orleans, La.

wave lengths, m, n, o, and p are the number of galvanometer divisions deflection per microgram of E or N added to the standards, and E and N are the quantities of E and N present in the unknown mixture.

This method of analysis requires, for correctness, that the fluorescence of a mixture equal the sum of that produced separately by the fluorescent substances present, and that fluorescence be proportional to concentration. Both requirements are met in the concentration range tested.

TABLE I. FLUORESCENCE OF HYDROXYPHENYL COMPOUNDS

SUBSTANCE	RING*	SIDE CHAIN	FILTER (Mμ)	RELATIVE FLUORESCENCE (%) pH_5	RELATIVE FLUORESCENCE (%) pH_{13}
l-Epinephrine	R_1-	$CHOHCH_2NH$ | CH_3	400	100.0	85.0
			436	100.0	76.0
DL-Isopropylnorepinephrine	R_1-	$CHOHCH_2NH$ | $CH(CH_3)_2$	400	72.0	--
			436	75.0	--
l-Norepinephrine	R_1	$CHOHCH_2NH_2$	400	85.0	71.0
			436	37.0	27.0
Threo-DL-3,4-dihydroxyphenylserine	R_1	$CHOHCHNH_2$ | $COOH$	400	0.19	0.08
			436	0.08	0.08
Erythro-DL-3,4-dihydroxyphenylserine	R_1-	$CHOHCHNH_2$ | $COOH$	400	0.03	0.01
			436	0.01	0.001
DL-Dopa	R_1-	CH_2CHNH_2 | $COOH$	400	0.051	5.0
			436	0.002	0.05
Dopamine	R_1-	$CH_2CH_2NH_2$	400	0.13	0.14
			436	0.04	0.04
3,4-Dihydroxyphenylacetic acid	R_1-	CH_2COOH	400	0.004	0.014
			436	0.002	(-)0.002
3,4-Dihydroxymandelic acid	R_1-	$CHOHCOOH$	400	(-)0.001	--
			436	(-)0.001	--
5-Hydroxytryptamine (serotonin)	R_2-	$3\text{-}CH_2CH_2NH_2$	400	0.022	0.11
			436	0.027	0.26

*R_1 = 3,4-dihydroxyphenyl; R_2 = 5-hydroxyindole.

RESULTS

1. *Fluorescence of Hydroxyphenyl Compounds.**—The fluorescence of certain compounds which occur biologically and which might interfere with the method is reported in Table I. All results are expressed in terms of the base or acid, and are referred to the fluorescence of adrenolutine (fluorescent product of epinephrine) at pH 5. Samples to be read at pH 5 were acidified by the addition of 2.0 ml. 5 N acetic acid 4 minutes after the addition of 20 per cent

*We wish to thank Dr. M. L. Tainter, Sterling-Winthrop Research Institute for supplying epinephrine, norepinephrine, and isopropylnorepinephrine; Dr. Richard Dahlbom, Astra Ltd., for supplying 3,4-dihyldroxyphenylacetic acid. Dr. M. D .Armstrong, University of Utah, for supplying 3,4-dihydroxymandelic acid; and Dr. W. G. Clark, Veterans Administration Center, Los Angeles 25, for supplying the dihydroxyphenylserines. Dopa, dopamine, and serotonin were obtained from the California Foundation for Biochemical Research.

sodium hydroxide. Blanks were prepared in an analogous manner. Each blank eluate contained an addition equal to that of the corresponding sample eluate. Additions of E, N, and isopropylnorepinephrine ranged between 0.02 and 0.10 μg; additions of the other substances varied from 50 to 100 μg.

Conversion of the sample pH from 13 to 5 reduced the fluorescence of dopa, 3,4-dihydroxyphenylacetic acid, and 5-hydroxytryptamine, but increased that of E and N. This procedure also reduced the blank readings. However, adrenolutine and noradrenolutine are unstable at pH 5, so that all readings must be made at a standard time after the addition of 20 per cent sodium hydroxide if the final pH is to be acid. In all subsequent analyses the pH has been adjusted to 5, the samples read at 7 and 10 minutes after the addition of sodium hydroxide, and the results averaged. This applies whether an eluate fraction is treated as a sample or as a blank.

2. *Error of Fluorometric Separation of E and N.*—Amounts ranging from 0.002 to 0.02 μg E and 0.005 to 0.1 μg N were added to blank column eluates, analyzed by the method described, and compared with eluates to which nothing had been added. The results are shown in Table II. Proportions ranging from 1:1 to 10:1 and amounts ranging from 0.1 to 0.002 μg could be separated fluorometrically with an average error of 4.6 per cent (range 0 to 14 per cent).

TABLE II. FLUOROMETRIC SEPARATION OF E AND N

μG ADDED		μG RECOVERED		PER CENT	
E	N	E	N	E	N
0.022	0.044	0.022	0.042	100	96
0.022	0.044	0.022	0.044	100	100
0.011	0.099	0.011	0.090	100	91
0.0055	0.0055	0.0063	0.0058	114	105
0.0055	0.0555	0.0054	0.0579	98	104
0.0022	0.0022	0.0020	0.0020	91	91

3. *Recovery of E and N by Alumina Column Chromatography.*—Amounts of E ranging from 0.12 to 0.002 μg were added to twenty-four 15 ml. samples of human and canine plasma which were then chromatographed on alumina. These eluates were analyzed and compared with others obtained from plasma to which nothing had been added, and the recovery calculated. The mean recovery was 94.2 per cent, with standard deviations of 10.5 per cent at the upper half of the range and 26.5 per cent at the lower. Seventeen additions of N ranging from 0.1 to 0.006 μg were recovered to the extent of 86.3 per cent with a standard deviation of 6.7 per cent at the upper half of the range and 20.2 per cent at the lower. There was no consistent difference between recoveries from canine and human plasma. The increased variation in apparent recovery observed when small additions are made to plasma is of the same order as the variation in replicates of a single sample, and thus probably represents the "background" variation in the analytic method which becomes apparent when low concentrations of E and N are analyzed rather than variation in the recovery from alumina.

4. *Evaluation of Blanks.*—Arterial plasma from 4 normal human volunteers and 2 dogs was allowed to stand at refrigerator temperature (4 to 7° C.) for 4 to 5 weeks, and then chromatographed on alumina columns. By this time more than 85 per cent of the N added to small fractions of the fresh plasma had disappeared.

Eluates of aged plasma were prepared as blanks by two procedures different from that described in the Method. Oxidation of the eluate at pH 6, followed by addition of NaOH, with addition of ascorbic acid 4 minutes later resulted in erratic blanks whose fluorescence occasionally exceeded that of other portions of the same eluate which had been treated as samples. Blanks prepared by omitting the oxidation step ($K_3Fe[CN]_6$ not added) were considerably lower than samples prepared from the same eluates.

For these reasons the technique outlined in the Method was used routinely to prepare blanks. Fluorescence was measured at pH 5. With this technique the difference between eluates of aged plasma treated as blanks and samples was small. Seven aged plasma samples averaged 0.023 μg/L. apparent E and 0.046 μg/L. apparent N. In eluates prepared from reagents only, there was no apparent E or N.

TABLE III. BLANK FLUORESCENCE AS PER CENT OF TOTAL

SUBSTANCE	AMOUNT ADDED μG	$100 \times \frac{\text{FLUORESCENCE OBSERVED IN BLANK WITH ADDITION}}{\text{FLUORESCENCE FROM SAME ADDITION TREATED AS A SAMPLE}}$
E	0.04	17
	0.04	14
	0.04	13
	0.04	24
	0.04	13
	0.04	29
	0.04	20
	0.02	6
	0.04	8
	0.04	21
	0.04	9
	0.02	17
	0.04	24
	0.04	25
		Average 17.1 ± 7.0 (s.d.)
N	0.04	2
	0.04	4
	0.04	4
	0.08	2
		Average 3.0 ± 1.1 (s.d.)

The blank values obtained by the method described are proportional to the E and N content of the sample (Table III). The explanation of this finding is that a small part of the E and N is changed, during adjustment of the pH to 6 with sodium hydroxide, to substances which subsequently form adrenolutine and noradrenolutine at high pH. The amount of noradrenolutine "preformed"

in the blanks is negligible (less than 5 per cent); that of adrenolutine can be reduced to about 15 to 20 per cent by careful titration with agitation. Too rapid titration or insufficiently thorough mixing during titration may yield blank values up to 40 per cent of the E present.

The over-all recovery of E or N equals the recovery from alumina multiplied by the recovery during the titration and oxidation steps. For E this is $100 \times 0.94 \times 0.83 = 78$ per cent, and for N is $100 \times 0.86 \times 0.97 = 83$ per cent. The apparent plasma concentrations of E and N reported below have not been corrected for these losses.

5. *Standard Deviation of a Single Determination.*—Four 30 ml. plasma samples were divided in half, and each fraction was separately chromatographed on an alumina column. Half of each eluate was treated as a sample, the other half as a blank. The standard deviation of a single determination was calculated as s.d. $= [^{Ed^2}/_{2n}]^{1/2}$, where d is the difference in estimated concentration between the duplicate analyses of each plasma sample, and n is the number of duplicates used in the calculation. The results were: s.d. of E determination = 0.042 μg/L. and of N = 0.13 μg/L.

6. *Normal Plasma Concentrations of E and N in Man.*—Thirty-milliliter blood samples were withdrawn from the brachial arteries or antecubital veins of 12 normal adults. In 7 cases the arterial and venous samples were withdrawn simultaneously. The subjects rested in the supine position during sampling and for 10 minutes before. All were hospital personnel (physicians, medical students, or nurses), and all appeared free from apprehension. The data are shown in Table IV. All concentrations are in terms of the bases, and are uncorrected for losses in recovery which average approximately twenty per cent (paragraph 4).

TABLE IV. NORMAL PLASMA E AND N CONCENTRATIONS ESTIMATED BY THE TRIHYDROXYINDOLE METHOD

SUBJECTS			SOURCE OF SAMPLE				DIFFERENCE	
			BRACHIAL ARTERY		ANTECUBITAL VEIN		A-V	
NO.	AGE	SEX	E (μg/L.)	N	E (μg/L.)	N	E (μg/L.)	N
1	25	F	0.00	0.25	0.05	0.34	(-)0.05	(-)0.09
2	26	M	0.13	0.18	(-)0.08	0.25	0.21	(-)0.07
3	23	M	(-)0.02	(-)0.08	0.06	0.11	(-)0.08	(-)0.19
4	23	F	0.25	0.25	0.00	0.46	0.25	(-)0.21
5	27	F	0.00	0.35	(-)0.07	0.43	0.07	(-)0.08
6	23	M	0.18	0.18	0.18	0.34	0.00	(-)0.16
7	26	F	0.18	0.26	0.02	0.58	0.16	(-)0.32
8	34	M	--	--	0.00	0.16	--	--
9	24	M	0.09	0.23	--	--	--	--
10	30	M	--	--	(-)0.01	0.19	--	--
11	28	M	--	--	0.01	0.48	--	--
12	24	M	--	--	(-)0.01	0.29	--	--
Average			0.10 ± 0.10 s.d.	0.20 ± 0.12 s.d.	0.01 ± 0.07 s.d.	0.34 ± 0.15 s.d.		

The levels of E in arterial plasma averaged 0.10 μg/L. and this concentration was significantly greater than zero ($p < 0.05$). The concentration of E in venous plasma averaged 0.01 μg/L. and was insignificantly different from

zero. The arteriovenous concentration difference of E was positive or zero in 5 of the 7 subjects from whom simultaneous samples were drawn, but a probably significant difference (0.16 μg/L. or more) was observed in only 3 cases, and the difference was statistically significant for neither the group as a whole nor for the A-V group.

The levels of N in venous plasma consistently exceeded those in arterial plasma. The difference in concentration averaged 0.14 μg/L. in the group as a whole and 0.16 μg/L. in the A-V group. The difference is statistically significant ($p < 0.01$) for the A-V group, but not for the group as a whole.

DISCUSSION

A serious limitation of chemical methods is that they do not establish the biologic activity of the substances estimated. Unfortunately, the concentrations of E and N present in human plasma are too low to permit estimation by the biologic assay methods currently employed. Holzbauer and Vogt[7] have shown the venous plasma of a single subject to contain less E and N than the minimum concentrations detectable by a sensitive biologic method, i.e., less than 0.06 μg/L. E and 1.0 μg/L. N. By the present (trihydroxyindole) method the concentration of E in venous plasma is insignificantly different from zero, while that of N is less than 0.6 μg/L. By the ethylenediamine method concentrations of E range from 0.1 to 1.3 μg/L. and of N from 2.1 to 5.2 μg/L. in normal human venous plasma[1, 3]; the reason for the variation in the results obtained is obscure. In addition to E and N, the ethylenediamine method is sensitive to the presence of dopamine, as well as to some of its supposed precursors and metabolites,[3] and probably to metabolites of N as well.

The trihydroxyindole method theoretically detects only 3,4-dihydroxyphenyl compounds with a two carbon side chain, a terminal nitrogen, and an hydroxyl group substituted on the β carbon atom.[8] Thus it should be insensitive to any of the hydroxyphenyl compounds, other than E and N, which are presently known to occur to an important extent in man or dog. Isopropylnorepinephrine, which could seriously interfere with the determination of E by the present method, may occur in the human adrenal medulla, but only in trace amounts.[9] Dopamine, which occurs in human urine, has been reported by von Euler and Floding[10] to produce fluorescence equal to 3 per cent of that of N by the trihydroxyindole method. With the present technique, the fluorescence of dopamine is approximately 0.1 per cent of that of N (Table I). A partial explanation of the difference lies in our exciting the samples at 400 mμ in addition to 436 mμ, and in reading the samples at pH 5, both of which increase sensitivity for N without appreciably modifying that for dopamine. However, a large part of the greater specificity of the present method is unexplained, and may be attributable to the optical system employed. It may also be pointed out that the fluorescences of serotonin and dopa at pH 13 are unduly high, and that measuring fluorescence at pH 5 causes a tenfold and a hundred fold decrease, respectively, in sensitivity for these substances.

The development of valid "blanks" for methods measuring endogenous substances is difficult. In the case where the substance analyzed is not normally present in the body blanks consist simply of analyses made before or at a suitable interval after administration of the foreign substance. In the present case, however, a theoretically ideal solution, in which the fluorescence developed by alkalinizing adrenochrome and noradrenochrome[4] is permitted to fall to zero by withholding ascorbic acid, appears inacceptable since the "blanks" frequently give higher readings than the unknowns. Obviously some aspect of the blanking technique causes one or more constituents of the eluates to emit or transmit more light than in the samples, and certainly the constituents concerned cannot be E or N. A large proportion of the "fluorescence" read by any fluorometer appears to result from reflected light (an empty cuvette gives a high reading), suggesting that the measurements must be a mixture of fluorometry, colorimetry, and nephelometry. In this circumstance the selection of a "blank" is to some extent arbitrary, since different solutions, none of which is fluorescent, may produce different readings in a fluorometer. We have retained the present blanking technique because it appears to be the only alternative to the original method[4] in which all constituents of the final mixture are present equally in samples and blanks. In the blanks, oxidation of E and N is prevented by the addition of ascorbic acid prior to the addition of ferricyanide, and consequently fluorescence does not develop when the mixture is made alkaline. A blanking method omitting ferricyanide entirely should achieve the same purpose, but we have rejected it because readings obtained by this method are consistently lower than when ferricyanide is added to the blanks. This is true even in plasma which has been blanked by aging, indicating that ferricyanide contributes to the "fluorescence" measured independently of its action on E or N.

The method described may be sufficiently sensitive to detect evidence of the resting secretion of the adrenal medullae, i.e., the difference between normal arterial plasma E concentrations and zero. This difference amounts to approximately 0.1 μg/L. which has been calculated previously[11] to result from the secretion of 0.1 to 0.2 μg/min. E from the adrenal medullae.

The significantly greater antecubital venous than arterial concentration of N presumably reflects the secretion of this substance from sympathetic nerves supplying the forearm. Studies are planned to determine whether a significant arteriovenous concentration difference also exists in organs such as brain, heart, and liver.

SUMMARY

1. A fluorometric method has been described which is capable of detecting as little as 1 Mμg of epinephrine and 2 to 3 Mμg of norepinephrine.

2. Of a series of biologically occurring substances of similar chemical structure, only isopropylnoradrenaline interfered with the detection of epinephrine or norepinephrine by this method. Isopropylnoradrenaline resembled epinephrine, but could be distinguished from norepinephrine.

3. The concentrations of epinephrine and norepinephrine in normal human arterial plasma were estimated as 0.10 ± 0.10 (s.d.) and 0.20 ± 0.12 (s.d.) micrograms per liter, respectively.

4. The concentration of epinephrine in antecubital venous plasma was lower, and that of norepinephrine higher, than in arterial plasma, the difference being statistically significant for norepinephrine but not for epinephrine.

REFERENCES

1. Weil-Malherbe, H., and Bone, A. D.: The Adrenergic Amines of Human Blood, Lancet **1**: 974-977, 1953.
2. Euler, C. v., Euler, U. S. v., and Floding, I.: Biologically Inactive Catechol Derivatives in Urine, Acta physiol. scandinav. **33** (Suppl. 118): 32-38, 1955.
3. Valk, A. de T., Jr., and Price, H. L.: The Chemical Estimation of Epinephrine and Norepinephrine in Human and Canine Plasma. I. A Critique of the Ethylenediamine Condensation Method, J. Clin. Invest. **35**: 837-841, 1956.
4. Lund, A.: Simultaneous Fluorimetric Determinations of Adrenaline and Noradrenaline in Blood, Acta pharmacol. et toxicol. **6**: 137-146, 1950.
5. Euler, U. S. v., and Floding, I.: A Fluorimetric Micromethod for Differential Estimation of Adrenaline and Noradrenaline, Acta physiol. scandinav. **33** (Suppl. 118): 45-56; 1955.
6. Ludeman, H. H., Filbert, M. S., and Cornblath, M.: Application of a Fluorimetric Method for Adrenaline-like Substances in Peripheral Plasma, J. Appl. Physiol. **8**: 59, 1955.
7. Holzbauer, M., and Vogt, M.: The Concentration of Adrenaline in the Peripheral Blood During Insulin Hypoglycaemia, Brit. J. Pharmacol. 9: 249-252, 1954.
8. Lund, A.: Fluorimetric Determination of Adrenaline in Blood. II. The Chemical Constitution of Adrenolutine (the Fluorescent Oxidation Product of Adrenaline), Acta pharmacol. et toxicol. **5**: 121-128, 1941.
9. Lockett, M. F.: Identification of an Isoprenaline-like Substance in Extracts of Adrenal Glands, Brit. J. Pharmacol. **9**: 498, 1954.
10. Euler, U. S. v., and Floding, I.: Diagnosis of Pheochromocytoma by Fluorimetric Estimation of Noradrenaline and Adrenaline in Urine, Scandinav. J. Clin. & Lab. Invest. **8**: 288-295, 1956.
11. Price, H. L.: Circulating Adrenaline and Noradrenaline During Diethyl Ether Anaesthesia in Man, Clin. Sc. **16**: 377-387, 1957.

THE EFFECT OF PRIOR CONTACT BETWEEN ACID CITRATE DEXTROSE AND SODIUM RADIOCHROMATE SOLUTIONS ON THE EFFICIENCY WITH WHICH Cr^{51} LABELS RED CELLS

T. A. CUNNINGHAM, M.B., M.R.C.P.E.,* E. M. McGIRR, B.Sc., M.B., M.R.C.P., AND W. E. CLEMENT, B.Sc.
GLASGOW, SCOTLAND

INTRODUCTION

SINCE Gray and Sterling[1] showed that human red cells contained about 20 μg per 100 ml. of chromium, Cr^{51}-labeled erythrocytes have been used to measure the body red cell mass and the in vivo red cell survival time. During experiments in this department to determine the optimum conditions for the tagging of red cells with Cr^{51} it was accidentally observed that the admixture of sodium chromate ($Na_2Cr^{51}O_4$) with an acid citrate dextrose (A.C.D.) solution before the addition of red cells (not after, as is the usual practice) led to comparatively poor labeling.

As such a finding appeared to preclude the preparation of stock mixtures of A.C.D. and $Na_2Cr^{51}O_4$ solutions in anticipation of their use, a series of experiments was designed to elucidate the reaction involved. While this work was in progress Cooper and Owen[2] reported impaired Cr^{51} labeling of erythrocytes when $Na_2Cr^{51}O_4$ solution was mixed with either A.C.D. solution or citrated plasma prior to the addition of blood. In explanation they postulated that "plasmatic reducing substances" converted the chromate to the chromic ion, which does not penetrate the red cell membrane but becomes bound to the plasma proteins.

Our investigations confirm that, under certain circumstances, reduction of the chromate ion does occur. They suggest that a chromic-citrate complex forms so that the chromic ion does not become bound to the plasma protein. The results appear to be of sufficient theoretical interest, as well as of enough practical importance to justify their publication.

METHODS

A preliminary experiment established that, at normal hematocrit values, more efficient labeling of red cells occurred in A.C.D. solution (1 part to 4 parts whole blood) than in saline suspension of red cells or when heparin was used as the anticoagulant (Fig. 1).

At lower hematocrit values, however, poorer labeling occurred in A.C.D. solution than in saline (Fig. 2).

From the University Department of Medicine, Royal Infirmary, Glasgow, Scotland.
Supported by the Board of Management, Royal Infirmary, Glasgow, and by the Rankin Fund of Glasgow University.
Received for publication, May 27, 1957.
*McIntyre Research Fellow, Royal Infirmary, Glasgow, Scotland.

With this knowledge the experiments were designed using A.C.D. anticoagulant and blood from patients with normal hematocrit values. Eight milliliters of whole blood or saline suspension of washed red cells were added to 2 ml. of the A.C.D. solution, or of the other test fluids, with which 5 μc of Cr^{51} had been mixed for varying periods of time. The mixtures were then incubated at 37° C. for 15 minutes. Thereafter, the red cells were separated by centrifugation for 5 minutes at 2,000 r.p.m. at a radius of 25 cm. and were washed once in normal saline. The red cells were hemolyzed by saponin before their radioactivity was assayed. Radioactivity was measured in an EKCO liquid scintillation counter, connected with an EKCO autoscaler. The sensitivity of the apparatus for Cr^{51} was approximately 14,000 c.p.m. per microcurie per 10 ml. with a background of 75 c.p.m. The uptake of Cr^{51} by the red cells was recorded by expressing the radioactivity of the red cells as a percentage of the total radioactivity of the cells and washings.

For the sake of brevity the essential details of the individual experiments are taken along with their results.

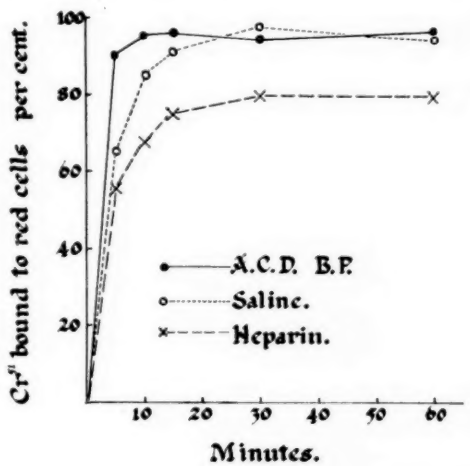

Fig. 1.

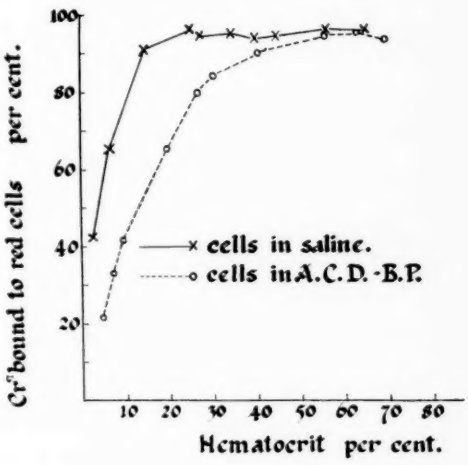

Fig. 2.

Fig. 1.—Comparison of efficiency of labeling of red cells with Cr^{51} in A.C.D. (B.P.), saline, and heparin.

Fig. 2.—Comparison of efficiency of labeling of red cells with Cr^{51} at different hematocrit values.

RESULTS

1. *The Effect of Prior Contact Between $Na_2Cr^{51}O_4$ and A.C.D. Solution.*—When $Na_2Cr^{51}O_4$ was left in contact with A.C.D. solution before the addition of blood, the labeling of the red cells was impaired. Fig. 3 demonstrates that the longer the contact the greater the deterioration in labeling efficiency. It also shows that deterioration is more rapid with A.C.D. (B.T.S.*) solution than with A.C.D. (B.P.†) solution.

2. *The Effect of Variation in Concentration of Sodium Citrate.*—Each of 5 solutions of disodium citrate, the citrate concentration such that it varied from 1.2 to 2.68 per cent after the addition of $Na_2Cr^{51}O_4$, was allowed to react for

*A.C.D. (B.T.S.) (Blood Transfusion Service): pH at 5.1 sodium acid citrate (2 per cent) and dextrose (3 per cent).

This solution is sterilized by autoclaving.

†A.C.D. (B.P.) (British Pharmacopeal Formula): pH at 5.1 trisodium citrate dihydrate (1.29 per cent), citric acid monohydrate (0.46 per cent), and dextrose (2.5 per cent).

These proportions of trisodium citrate and citric acid yield in solution disodium hydrogen citrate. The citrate and dextrose solutions are prepared and are autoclaved separately. The final mixture is resterilized by Seitz filtration.

1 hour. In all of these solutions, especially the more concentrated, labeling was impaired compared with controls in which the Cr^{51} was added after the blood (Table I).

3. *The Effect of Citrate Solution of Varying pH.*—Equimolar solutions (0.0847 M) of mono-, di-, and trisodium citrate of pH 4, 5.1, and 8, respectively, were allowed to react for 1 hour with the $Na_2Cr^{51}O_4$ solution. Labeling of the red cells was impaired in the more acid solutions (Table II).

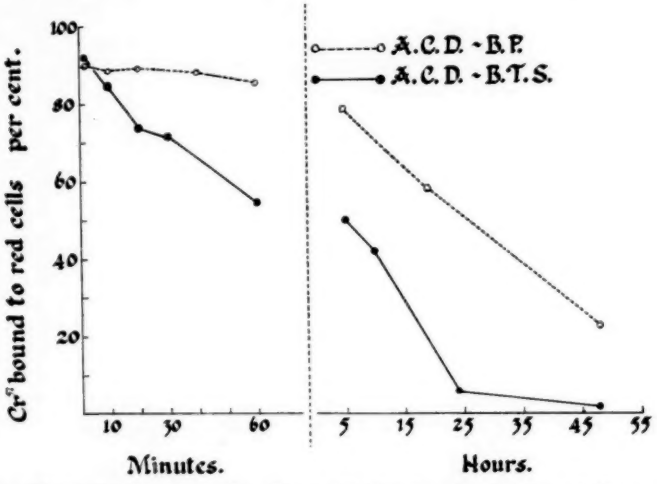

Fig. 3.—The effect of prior contact between $Na_2Cr^{51}O_4$ and A.C.D. solutions on the efficiency of labeling of red cells with Cr^{51}.

TABLE I. THE EFFECT OF VARIATION IN CONCENTRATION OF SODIUM CITRATE ON THE EFFICIENCY OF LABELING RED CELLS WITH Cr^{51}

CONCENTRATION OF CITRATE (PER CENT)	PER CENT Cr^{51} BOUND TO RED CELLS	
	PRIOR CONTACT BETWEEN $Na_2Cr^{51}O_4$ AND CITRATE	
	1 HOUR	NIL
1.2	77	96
1.6	79	96
2.0	72	95
2.4	65	95
2.68	49	93

TABLE II. THE EFFECT OF VARIATION IN pH OF CITRATE SOLUTIONS ON THE EFFICIENCY OF LABELING RED CELLS WITH Cr^{51}

SOLUTION	pH	PER CENT Cr^{51} BOUND TO RED CELLS	
		PRIOR CONTACT BETWEEN $Na_2Cr^{51}O_4$ AND CITRATE	
		1 HOUR	NIL
Monosodium citrate	4.0	60	87
Disodium citrate	5.1	76	89
Trisodium citrate	8.0	91	93

4. *The Effect of Variation in Concentration of Dextrose.*—Five solutions of dextrose (unbuffered), of which the final concentration after the addition of $Na_2Cr^{51}O_4$ solution was 1.2 to 2.68 per cent, were allowed to react for 1 hour. They produced slight but consistent impairment of labeling compared with simultaneous controls (Table III).

5. *The Effect of Dextrose Solutions of Varying pH.*—Solutions of dextrose of 2.4 per cent final concentration and pH of 2, 6, and 10 were allowed to react with the $Na_2Cr^{51}O_4$ solution for 19 hours. The pH of the mixture was adjusted to approximately 7 before the addition of red cells. Control experiments for each pH were done with saline in place of dextrose. The dextrose solutions diminished the ability of the Cr^{51} to label red cells. This effect was most pronounced in acid solution. Less pronounced loss of labeling was observed in the saline controls (Table IV).

TABLE III. THE EFFECT OF VARIATION IN CONCENTRATION OF DEXTROSE ON EFFICIENCY OF LABELING RED CELLS WITH Cr^{51}

CONCENTRATION OF DEXTROSE (PER CENT)	PER CENT OF Cr^{51} BOUND TO RED CELLS	
	PRIOR CONTACT BETWEEN $Na_2Cr^{51}O_4$ AND DEXTROSE	
	1 HOUR	NIL
1.2	87	92
1.6	87	92
2.0	87	93
2.4	86	91
2.68	84	90

TABLE IV. THE EFFECT OF VARIATION IN pH OF DEXTROSE SOLUTIONS ON THE EFFICIENCY OF LABELING RED CELLS WITH Cr^{51}. PRIOR CONTACT BETWEEN $Na_2Cr^{51}O_4$ AND DEXTROSE OF 19 HOURS

pH OF DEXTROSE SOLUTION	PER CENT OF Cr^{51} BOUND TO RED CELLS	
	DEXTROSE	SALINE CONTROL
2	0	37
6	21	91
10	65	81

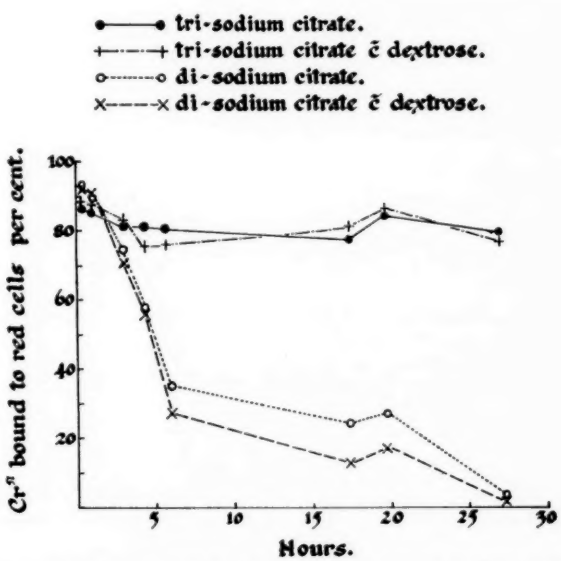

Fig. 4.—The effect of prior contact between $Na_2Cr^{51}O_4$ and citrate solutions of pH 5.1 and 8, with and without glucose, on the efficiency of labeling of red cells with Cr^{51}.

6. *The Effect of Citrate Solutions With and Without Glucose.*—Solutions of sodium citrate of pH 5.1 and 8, respectively, were used, both with and without

3 per cent dextrose. When the $Na_2Cr^{51}O_4$ was left in contact with these solutions, the ability of the Cr^{51} to label red cells was impaired especially at the lower pH (Fig. 4). The longer the exposure the more noticeable was the effect which was probably also more obvious in the presence of dextrose.

7. *The Effect of Light on the Reaction Between the $Na_2Cr^{51}O_4$ and A.C.D. Solutions.*—As chromates have photosensitive properties, it was thought worth while to investigate the effect of light on the reaction between $Na_2Cr^{51}O_4$ and A.C.D. solution.

$Na_2Cr^{51}O_4$ was added to A.C.D. (B.P.) solution and part of the mixture kept in the dark while the remainder was exposed to the light of a 100 Watt tungsten lamp at a distance of 6 inches. The temperature of both solutions was controlled at 18° C. by means of a cirulating water bath. Assays of the labeling power of these mixtures at intervals showed that in the dark there was little impairment in the first 4 hours but in the light rapid loss occurred from the outset (Fig. 5).

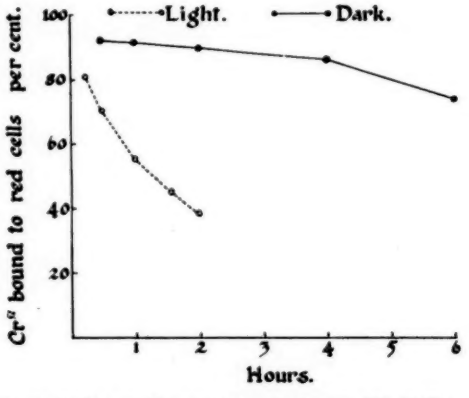

Fig. 5.—The effect of light on the reaction between $Na_2Cr^{51}O_4$ and A.C.D. solutions.

8. *The Effect of Autoclaving A.C.D. Solution.*—When A.C.D. solution was heated to 100° C. for 4 hours a yellow discoloration developed, indicative of early caramelization of the dextrose. The labeling power of $Na_2Cr^{51}O_4$ was impaired very rapidly when mixed with these solutions. (Table V).

TABLE V. THE EFFECT OF AUTOCLAVING A.C.D. SOLUTIONS ON THE EFFICIENCY OF LABELING RED CELLS WITH Cr^{51}

DURATION OF CONTACT BETWEEN $Na_2Cr^{51}O_4$ AND A.C.D.	PER CENT OF Cr^{51} BOUND TO RED CELLS			
	A.C.D. (B.P.)		A.C.D. (B.T.S.)	
	AUTOCLAVED	NOT AUTOCLAVED	AUTOCLAVED	NOT AUTOCLAVED
30 min.	3	84	2	86
60 min.	2	81	2	88

9. *Attempts to Identify the Degradation Products of Sodium Chromate Formed in the Preceding Reaction.*—Evidence for the reduction of the chromate ion to the chromic ion was found. $Na_2Cr^{51}O_4$ solution was exposed to the action of 3 per cent dextrose in N/10 HCl, both in the light and in the dark

for 4 hours. Any chromic ions formed by reduction from chromate were then precipitated by ammonia. Carrier chromic ions were provided to facilitate handling of the precipitate and carrier chromate to minimize the loss of unreduced chromate due to adsorption by the fine precipitate. The precipitate was separated and redissolved in HCl so that its radioactivity could be estimated. The precipitation, along with the carrier chromic ion, of a proportion of the radioactivity, which increased with the passage of time and on exposure to light, indicated that, under the conditions of this experiment, chromate was reduced by dextrose (Table VI).

TABLE VI. PERCENTAGE OF Cr^{51} PRECIPITATED WITH CHROMIC IONS AFTER ADMIXTURE OF $Na_2Cr^{51}O_4$ WITH 3 PER CENT DEXTROSE IN N/10 HCL

DURATION OF CONTACT BETWEEN $Na_2Cr^{51}O_4$ AND 3 PER CENT DEXTROSE IN N/10 HCl	SOLUTION EXPOSED IN	
	LIGHT	DARK
1 Hour	38	14
4 Hours	59	31

The formation of complexes between chromic ions and citrate precluded the use of this method to study the reaction between $Na_2Cr^{51}O_4$ and citrate solutions, or for that matter the use of the ion exchange resin technique.

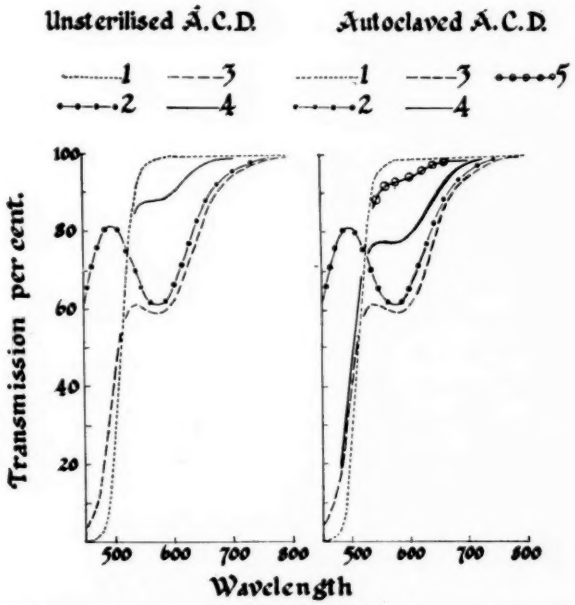

Fig. 6.—The effect of autoclaving A.C.D. (B.P.) solution on the rate at which it reacts with 1 per cent K_2CrO_4 solution. Curves 1, 2, and 3 are the transmission spectra of freshly prepared mixtures of A.C.D. (B.P.) solution with 1 per cent K_2CrO_4, 1 per cent $Cr_2(SO_4)_3$, and 0.5 per cent K_2CrO_4 plus 0.5 per cent $Cr_2(SO_4)_3$. Curve 4 is the transmission spectrum of 1 per cent K_2CrO_4 solution mixed with A.C.D. (B.P.) solution after 11 days' exposure to light. Curve 5 is the same for a similar mixture kept in the dark for 11 days.

Instead a spectrophotometric analysis was made of mixtures of 1 per cent K_2CrO_4 with acid dextrose (N/10 HCl), A.C.D. (B.P.), citric acid, monosodium citrate, and disodium citrate solutions. The same method was used to

study the reaction between K_4CrO_2 and A.C.D. (B.P.) solution, and between K_2CrO_4 and 3 per cent dextrose in 2 per cent acid citrate solution, before and after autoclaving.

Solutions which contain chromic ions have an absorption band maximal between 570 and 580 mμ. An absorption band in this position developed in the test solutions except in the mixtures with disodium citrate where little or no reaction was observed. The absorption band became more intense with time, the change being more rapid in solutions exposed to light. When solutions containing dextrose were autoclaved, the appearance of the absorption band was more rapid than in identical solutions which were not autoclaved (Fig. 6).

DISCUSSION

With the standard technique used in our department excellent labeling of red cells occurs. Four parts of whole blood are mixed with 1 part of A.C.D. solution and $Na_2Cr^{51}O_4$ is then added. The mixture is incubated at 37° C. for 15 minutes. Provided that the hematocrit is 40 per cent or more approximately 90 per cent of the Cr^{51} is taken up by the red cells. In saline suspensions of red cells, even when the hematocrit of the mixture is as low as 20 per cent, equally good labeling occurs. Cooper and Owen[2] reported similar efficient labeling in A.C.D. solution with blood of normal hematocrit values. They also noted diminution of the labeling efficiency as the hematocrit fell. They observed that labeling with Cr^{51} never reaches 100 per cent, a finding which they attributed to the conversion, by "plasmatic reducing substances" of a small amount of the chromate ion to the cationic chromic state, in which they considered it would become bound to the plasma protein.

The details of their experiments, in which they studied the reduced efficiency in labeling red cells when the chromate solution was premixed with plasma, show that the Cr^{51} was exposed not to plasma only but to a plasma-citrate-dextrose mixture. In the light of our experience it would appear that at least part of the effect they observed could be attributed to the citrate-dextrose mixture rather than to the plasma.

The impaired labeling in A.C.D. solution of blood samples with low hematocrit values is probably due to the fact that under these conditions the chance is reduced of a chromate ion entering a red cell and becoming bound there before being affected by the citrate-dextrose in the suspending solution. In saline suspensions of red cells, where no component of the suspending solution is capable of reacting with the chromate ion, a very large proportion of these ions will enter the red cells even when these are widely spaced in anemic blood.

Our experience indicates that the blood of anemic patients can be effectively labeled in three ways. The hematocrit may be adjusted to 40 or more by the removal of some of the plasma, or the cells may be washed and suspended in saline before the addition of the $Na_2Cr^{51}O_4$. Finally a larger dose of $Na_2Cr^{51}O_4$ may be added to the anemic blood, to allow for less efficient labeling, and the surplus radioactivity removed by washing the cells after incubation.

The accidental observation that admixture of $Na_2Cr^{51}O_4$ with A.C.D. solution before the addition of red cells might lead to comparatively poor labeling is amply confirmed in the experiments designed to elucidate this finding. They show that a variety of influences may impair the ability of the chromate ion to enter red cells. The labeling power of $Na_2Cr^{51}O_4$ is adversely affected by decreasing the pH of the solution, by increasing the citrate concentration, and to a lesser extent by increases in concentration of dextrose. Whatever the nature of the reaction involved, it is accelerated by exposing the reaction mixture to light. Impairment occurs more rapidly with A.C.D. (B.T.S.) solution than with A.C.D. (B.P.) solution. The concentrations of both citrate and dextrose are greater in A.C.D. (B.T.S.) solution than in A.C.D. (B.P.) solution, and these differences are probably of significance. The methods of sterilizing these solutions also differ in detail. Autoclaving dextrose solution tends to cause caramelization with an increase in its reducing properties. The components of both A.C.D. preparations are autoclaved but it would appear that the technique used by the B.T.S. laboratory causes more caramelization. We have demonstrated that A.C.D. (B.P.) solution is also capable of causing very rapid deterioration in the labeling power of $Na_2Cr^{51}O_4$ if visible caramelization is produced in it by heating to 100° C. for 4 hours.

Anionic chromium in the form of $Na_2Cr^{51}O_4$ has a marked affinity for red cells and in the tracer amounts used in labeling techniques, under optimum conditions, it is almost entirely taken up by the red cells. Cationic chromium in the form of $Cr^{51}Cl_3$, on the other hand, enters the red cells only in negligible amounts, if it does so at all. Thus reduction of the chromate to the chromic ion, as suggested by Cooper and Owen[2] offers a simple but probable explanation for the changes in $Na_2Cr^{51}O_4$ when it is mixed with A.C.D. before the addition of red cells. Evidence that reduction of chromate to chromic ions by acid dextrose does occur was obtained both by tracer techniques and by spectrophotometric analysis of mixtures of acid dextrose with K_2CrO_4.

The formation of complexes between chromic ions and citrates precludes the use of the more direct methods of detecting the conversion of chromate to chromic ions, such as precipitation or trapping by an ion exchange resin, to study the reaction between $Na_2Cr^{51}O_4$ and citrate solutions. However, the spectrophotometric analysis of mixtures of citrate and chromate solutions reveals, in time, the development of an absorption band between 570 and 580 mμ; this is of similar configuration to that found in the test solutions to which chromic ions have been added. The rate of development of this band appears to be influenced by the same factors as those which affect the labeling power of chromate. Thus it appears more rapidly in acid solutions exposed to the light and when the A.C.D. solution has been partially caramelized by heating. There is a discrepancy in the case of disodium citrate which proved inactive against 1 per cent K_2CrO_4 in the spectrophotometric study, whereas in the tracer experiments it was almost as active as A.C.D. solution in reducing the labeling power of chromate. This may well be explained by the buffering

action of 1 per cent K_2CrO_4 solution which is probably sufficient to raise the pH of the disodium citrate solution to a value at which the reaction is very slow. The critical value for this purpose would appear to be about pH 6. At lower values a proportion of the chromate is converted to the more active dichromate and chromic acid.

The difficulty which we experienced in measuring the proportion of chromate ion reduced to the cationic state in citrate solutions raises the question of whether the biologic behavior of the chromic-citrate complex may differ from that of the free chromic ion which, as previously mentioned, becomes bound to the plasma proteins. Pilot experiments have been carried out to clarify this question. Serum was incubated with chromic chloride or with chromate, the power of which to label red cells had been destroyed by contact with A.C.D. solution. Electrophoretic analysis of the serum showed that the chromic chloride and the reduced chromate behave differently. Chromic chloride moves with the proteins, chiefly the albumin fraction, whereas the "reduced chromate" migrates very rapidly towards the anode, behaving as a free anion. Comparison of the behavior of chromic chloride and "reduced chromate" in vivo would be of interest.

CONCLUSIONS

Our studies have shed light on the change that occurs in the chromate ion when solutions of $Na_2Cr^{51}O_4$ and A.C.D. are mixed. There is good evidence that the chromate ion is reduced to the chromic state so that it can no longer enter the red cells, as suggested by Cooper and Owen.[2] Our investigations also suggest that a chromic-citrate complex forms, the biologic behavior of which is as yet unknown. Pilot electrophoretic studies show that chromic chloride and the chromic-citrate complex behave differently in vitro. The former moves with the proteins, whereas the latter migrates rapidly towards the anode.

Our experience suggests that it is not possible to keep stock mixtures of A.C.D. and $Na_2Cr^{51}O_4$ solutions for more than 4 hours, if efficient labeling is to be maintained. Even this period of premixing is possible only if the A.C.D. solution is not sterilized by autoclaving, and the mixture is not exposed to strong light.

SUMMARY

Prior admixture of $Na_2Cr^{51}O_4$ and A.C.D. solutions, which would enable a solution of A.C.D. labeled with Cr^{51} to be maintained for emergency use, impairs the ability of Cr^{51} to tag red cells.

The effect is more obvious with A.C.D. (B.T.S.) than with A.C.D. (B.P.) solution.

Labeling is adversely affected by the duration of prior mixing before the addition of red cells, by decreasing the pH of the solutions, by increasing the concentration of citrate and dextrose, by exposing the A.C.D and $Na_2Cr^{51}O_4$ mixture to light, and by autoclaving the dextrose solutions.

Inquiry into the nature of the change which occurs in the $Na_2Cr^{51}O_4$ has established that the chromate is reduced to the chromic ion. The "reduced chromate" forms a firm complex with citrate and does not become bound to the plasma protein as does the free chromic ion. Evidence is presented to show that it is not possible to keep a solution of A.C.D. labeled with Cr^{51} and maintain efficient labeling for more than 4 hours.

We wish to express our thanks to Professor L. J. Davis for his advice and criticisms. We are grateful for the help of Dr. Bell, Department of Chemistry, Glasgow University, and Dr. Crawford, Royal College of Science and Technology, Glasgow, with whom we discussed the chemical aspects of the investigations.

The radiochromium was supplied by the Regional Physics Centre, Glasgow, Scotland.

REFERENCES

1. Gray, S. J., and Sterling, K.: The Tagging of Red Cells and Plasma Proteins With Radioactive Chromium, J. Clin. Invest. **29**: 1604-1613, 1950.
2. Cooper, M., and Owen, C. A.: Labeling Human Erythrocytes With Radiochromium, J. LAB. & CLIN. MED. **47**: 65-71, 1956.

PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

Thirtieth Annual Meeting

Chicago, Ill., Nov. 1 and 2, 1957

ABSTRACTS

1. COMPARISON OF THE INFLUENCE OF OPERATIVE TRAUMA AND NUTRITIONAL MANAGEMENT ON METABOLIC ALTERATIONS

WILLIAM E. ABBOTT, M.D., HARVEY KRIEGER, M.D. (BY INVITATION), AND STANLEY LEVEY, PH.D. (BY INVITATION), CLEVELAND, OHIO

In previous studies on patients of the same sex undergoing a gastrectomy it was noted that by providing adequate amounts of nitrogen, calories, electrolytes, and fluids, postoperative abnormalities in the metabolism of sodium, nitrogen, and potassium as well as loss of body weight could be minimized or negated. In order to eliminate the effect of variations resulting from different ages and states of nutrition and to assess the effect of operative stress versus diet, the following studies were carried out. The metabolic changes resulting from a 3-day control period of intravenous feeding were compared with alterations occurring following cholecystectomy when the patients were maintained on the same intravenous regimen as that given during the control period. Ten women were studied, all of whom underwent a cholecystectomy. Three were maintained solely on a 5 per cent dextrose solution in water during the control and 3-day postoperative period. Three additional subjects received a comparable regimen plus approximately 90 mEq. of both sodium and potassium. A third group of 4 women were given the latter regimen plus a 5 per cent protein hydrolysate. The daily excretion of urinary 17-hydroxycorticoids were within the normal range for both the control and oral repletion period. On the day of operation and the next two days there was an increase in excretion of the 17-hydroxycorticoids (50 to 300 per cent of the control value).

It may be concluded from the results obtained that while there was some variation from patient to patient in the metabolic alterations of the two periods the changes noted were largely due to the nutritional regimen employed and to a lesser degree to the stress of operation. Similar studies with another group of patients indicate that postoperative complications (hemorrhage, fever, etc.) or severe trauma in men evokes a greater metabolic response than that occurring in these women who underwent a cholecystectomy.

2. RELATIONSHIP BETWEEN A RANGE OF TISSUE TEMPERATURE AND LOCAL OXYGEN CONSUMPTION IN THE RESTING FOREARM

D. I. Abramson, M.D., A. Kahn (By Invitation), S. Tuck, Jr., B.S. (By Invitation), G. A. Turman, M.D. (By Invitation), H. Rejal, B.S. (By Invitation), and C. J. Fleischer, M.D. (By Invitation), Chicago, Ill.

With the introduction of hypothermia in surgery interest has been revived in the effects of temperature on tissue metabolism. Although work has been performed in the past on in vitro muscle slices and on the entire organism, the effects of changes in temperature on the oxygen consumption of specific tissues in vivo have not been reported. Such a study was attempted in the present investigation, involving 21 experiments on 13 normal subjects.

In each instance blood flow in the forearm was obtained using the venous occlusion plethysmograph, with the limb being exposed consecutively to water at 45°, 32°, and 17° C. Oxygen content of samples of blood, collected at each bath temperature from veins draining the tissues under study, was determined with the Van Slyke manometric method. Skin, subcutaneous tissue, and muscle temperatures were also recorded at each bath temperature, using thermocouples. From the data on blood flow and oxygen determination, oxygen consumption of the tissues of the forearm (except bone) was calculated.

At an average tissue temperature of 39.9° C. (produced by a bath temperature of 45° C.), the average oxygen consumption was 0.332 ml./min./100 ml. limb volume. At an average tissue temperature of 34.5° C. (produced by a bath temperature of 32° C.), the average oxygen consumption was 0.173 ml. At an average tissue temperature of 24.0° C. (produced by a bath temperature of 17° C.), the average oxygen consumption was 0.071 ml.

A statistical analysis of the variations of oxygen consumption as related to average tissue temperature revealed an overwhelmingly significant difference in the readings obtained at the three bath temperatures.

Therefore it was concluded that exposure of the tissues of the forearm to local heat produced a definite rise in oxygen consumption, while cold had the opposite effect. The Q_{10} of the oxygen consumption rate was found to have a mean value of 2.5.

3. EXOPHTHALMOMETRY IN GRAVES' DISEASE

A Twenty-year Study

Willard Bartlett, Jr., M.D., St. Louis, Mo.

Exophthalmometric measurements are reported on 75 patients treated for Graves' disease since 1936. The average period of serial observations is 11 years, the shortest is 2 years, and the longest is 20 years.

Analysis of Data on Proptosis.—(1) No characteristic change accompanied the uniform metabolic improvement during preoperative treatment. (2) Between preoperative and early (4 months) postoperative measurements, decrease occurred in 10 per cent. (3) Between either preoperative or early postoperative measurement and the final observation, decrease occurred in 16 per cent, increase

in 74 per cent. (4) The range of final measurements in 59 instances reveals proptosis of 18 mm. or less (bilateral) in 15 per cent, of 20 mm. or less in 27 per cent. (5) In serial observations on 46 postoperative patients of constant therapeutic regimens, variation of proptosis of as little as 1 mm. was seen in only 2 patients, regardless of metabolic status.

Comment.—Individuals who have had Graves' disease show a wider variation of proptosis from time to time than do others. Cosmetic improvement reflects only narrowing of the palpebral fissure. Postoperative hypothyroidism is accompanied by exaggeration of the usual increase in proptosis, and this is prevented or reversed by adequate thyroid dosage, as stated by Ruedemann (1931) and the author (1932), even in early "malignant" exophthalmos.

These data do not support the views that there is a correlation between increase of proptosis and either the rapidity (Soley) or the amount (Dobyns) of reduction of metabolism.

Undoubtedly, many orbital decompressions have been performed without knowledge of an alternative. In this series, 5 patients with "malignant" exophthalmos have been seen and 4 have previously been cared for, none requiring orbital decompression since 1930.

Desiccated thyroid in small doses is being employed late in the preoperative regimen and is promptly increased after operation to maintenance dosage. Children are treated exactly as are adults. The author continues to reject the dictum that thyroidectomy should be avoided in the patient with severe exophthalmos and mild hyperthyroidism.

Menstrual histories of 50 patients in this series suggest strongly that menstruation persists to a higher age in women who have had Graves' disease than in others.

4. PULMONARY FUNCTION IN OBESE PERSONS

GEORGE N. BEDELL, M.D. (BY INVITATION), WILLIAM R. WILSON, M.D. (BY INVITATION), AND PAUL M. SEEBOHM, M.D., IOWA CITY, IOWA

Numerous medical reports have appeared calling attention to a syndrome of obesity, arterial hypoxemia, alveolar hypoventilation, and polycythemia. This study was undertaken to demonstrate (1) the frequency of this syndrome in extremely obese persons, and (2) the mechanism of hypoxemia. Pulmonary function studies have been done in 22 obese persons and 14 of these have been restudied following weight loss.

The patients are divided into 2 groups depending on arterial oxygen saturation at rest. Twelve patients (average weight 300 pounds) with normal saturation were put in Group I. These patients had normal lung volumes; mean minute volume of ventilation of 9.9 L., mean alveolar ventilation of 6.3 L., mean physiologic dead space of 215 ml., slightly uneven distribution of inspired air, mean maximal breathing capacity (MBC) of 86 per cent of predicted normal; mean maximal expiratory flow rate (MEFR) of 209 L./min., mean maximal inspiratory flow rate (MIFR) of 154 L./min., and normal diffusion. Five patients restudied after a mean loss of 54 pounds had an increase in mean expiratory reserve volume (ERV) from 64 per cent to 106 per cent, increase in mean MBC from 76 per cent to 91 per cent, increase in mean MEFR from 180 L./min. to 199 L./min., and MIFR from 170 L./min. to 194 L./min.

Ten obese patients (average weight 315 pounds) with arterial hypoxemia compose Group II. Nine of the 10 had clinical or laboratory evidence of lung

disease. One patient had arterial hypoxemia (90.2 per cent) and no evidence of lung disease. Following weight reduction some of these patients had improved pulmonary function.

We conclude that in most obese persons hypoxemia is secondary to lung disease and not caused by obesity alone. Obesity in persons with normal lungs causes a decrease in ERV, MBC, MEFR, and MIFR, but is partly reversible by weight reduction.

5. OBSERVATIONS ON POTASSIUM REQUIREMENTS IN MAN

WILLIAM B. BEAN, M.D., AND ROBERT HODGES, M.D. (BY INVITATION),
IOWA CITY, IOWA

There are no long-term studies of potassium requirements in man. Often it is stated that any natural diet has enough potassium. In recent experiments on pantothenic acid metabolism, 6 normal young men were maintained on a purified, partly synthetic diet containing 800 mg. of potassium a day expressed as potassium ions. Three of the 6 received an additional gram of potassium chloride daily. One patient with added potassium and one without were given only the standard diet and a standard vitamin and mineral supplement. Another pair had the same regimen but pantothenic acid was removed, and the remaining pair had large doses of a pantothenic acid antagonist omegamethyl pantothenic acid. The experimental period of 10 weeks followed a 4-week control period. There was a similar recovery period. The urinary excretion of potassium fell rapidly in all subjects on the experimental diet and became steady at a lower level after two weeks, ranging from 10 to 20 mEq. a day. On many occasions the serum potassium of those without supplements fell below our lower limit of normal, but symptoms of hypokalemia did not appear. Serum potassium remained just at or below the lower limits of normal. In each subject with supplemental potassium, the urinary levels were a little higher and the blood levels within the normal range. Though there was a slight rise in blood CO_2, alkalosis did not develop.

These observations indicate that an intake of 800 mg. of potassium ions is about the minimal daily requirement, and that an additional gram a day is adequate, and perhaps ample.

6. METABOLIC EFFECTS OF HUMAN AND MONKEY GROWTH HORMONE IN MAN

DELBERT M. BERGENSTAL, M.D., HERBERT A. LUBS, M.D. (BY INVITATION), LOIS F. HALLMAN (BY INVITATION), JAMES PATTEN, M.D. (BY INVITATION), HERBERT J. LEVINE, M.D. (BY INVITATION), AND C. H. LI, M.D. (BY INVITATION),
BETHESDA, MD.

Growth hormone prepared from human and monkey pituitary glands by C. H. Li has been shown to have differences in chemical structure and physical properties from that prepared from beef pituitary glands. These growth hormone preparations were administered to 3 patients under metabolic balance conditions. At dose levels from 2.5 to 10 mg. a day, marked metabolic effects were observed including positive nitrogen, phosphorus balances, and altered electrolyte balances. By use of intravenous glucose-insulin tolerance tests a definite insulin resistance was observed. One patient with persistent lactation

demonstrated a marked increase in lactation during growth hormone administration. A comparative study of monkey and human growth hormone was made in a hypopituitary patient which showed greater activity of the human preparation than the monkey preparation. The details of the metabolic balance results will be presented. There was no evidence of adrenocortical stimulation and only slight thyroid stimulation by these hormones. Measurement of aminopolysaccharides in the urine in a hypophysectomized patient with chondrosarcoma resulted in an increase of this material in the urine during growth hormone administration and this probably reflects a stimulation of the formation of mucopolysaccharides by the tumor since cartilage tissue is extremely sensitive to growth hormone. The relationships of the differences in structure between beef and human growth hormone in reference to its metabolic activity will be discussed.

7. AN ATHYROTIC MOTHER WITH TWO CRETINOUS CHILDREN WITH THYROID GLANDS

WILLIAM H. BEIERWALTES, M.D., E. A. CARR, JR., M.D. (BY INVITATION), V. N. DODSON, M.D. (BY INVITATION), G. H. LOWREY, M.D. (BY INVITATION), A. H. WHEELER, PH.D. (BY INVITATION), AND N. SPAFFORD, A.B. (BY INVITATION), ANN ARBOR, MICH.

Most cretins are athyrotic and their mothers are euthyroid. The cause of thyroid defects resulting in cretinism is unknown. To our knowledge, no one has reported sibling cretins with thyroid glands born to an athyrotic mother, nor has anyone suggested that cretins might acquire thyroid damage from maternal or fetal thyroid autoantibodies.

A clinically hypothyroid 26-year-old mother and her 3-year-old and 2-year-old cretins were studied. The mother's 1-, 2-, 5-, and 24-hour thyroid I^{131} uptakes, all under 3 per cent, the scintiscan of her neck, and her B.M.R. of –32 per cent were compatible with total absence of thyroid gland. She regularly had taken 90 to 192 mg. of thyroid per day throughout both pregnancies. The cretins, at University Hospital before 6 months of age, had demonstrated classic physical and roentgenographic stigmata of cretinism, hypothyroid I^{131} uptakes, and a classic response to the subsequent administration of desiccated thyroid. Off thyroid for 6 weeks, the children both attained normal I^{131} uptakes during the 1- to 5-hour intervals but subnormal 24-hour uptakes. The 3-year-old demonstrated an elevated PBI^{131} at 24 hours, and the 2-year-old a high normal PBI and low normal serum cholesterol. Paper chromatography of serum after I^{131} demonstrated significant quantities of thyroxine in both cretins, triiodothyronine in the younger and no diiodotyrosine in either. Precipitin and complement fixation tests of the sera of this mother and these children and of 11 other mothers of 11 other proved cretins 6 weeks to 13 years of age have not demonstrated human thyroid antoantibodies to date. We have, however, demonstrated high human thyroid autoantibody titers in human Hashimoto's struma, and we have demonstrably "immunized" rabbits to human thyroid antigen.

These findings suggest that: (1) cretinism in the human is not a result of a low level of maternal thyroid hormone; (2) a child may be born with "normal" but too little thyroid gland to maintain adequate body growth; and (3) there is no proof, to date, that thyroid autoantibodies of mother or child are the cause of thyroid defects of cretinism.

8. OBSERVATIONS ON INTRINSIC FACTOR AND THE INTESTINAL ABSORPTION OF VITAMIN B_{12}

WILLIAM R. BEST, M.D., JOHN H. FRENSTER, M.D. (BY INVITATION), AND MARVIN M. ZOLOT, M.D. (BY INVITATION), CHICAGO, ILL.

$B_{12}Co^{60}$ can be absorbed when placed by tube with intrinsic factor concentrate (IFC) in the stomach, duodenum (best absorption), jejunum, or ileum (Fed. Proc. **16**: 353, 1957). A patient with pernicious anemia had 8.9 per cent 24-hour urinary excretion when 1.0 μg $B_{12}Co^{60}$ was given with 1.0 U. S. P. unit IFC by tube into the proximal ileum followed by 1.0 mg. B_{12} parenterally. Peak excretion of 1.05 per cent/hr. occurred at 8 to 10 hours, the same time as after oral administration. *Thus, the main delay in transport of B_{12} from stomach to blood stream is not due to intestinal transit time, but must be related to mucosal cell processes.* When $B_{12}Co^{60}$ was similarly given into the mid-jejunum without IFC, only 0.6 per cent was excreted, confirming that protection of B_{12} from inactivating forces high in the intestine is not the mode of IFC action.

Another PA patient received 0.5 unit IFC with 1.0 μg $B_{12}Co^{60}$ and 100 ml. thin barium through a tube just proximal to a balloon obstructing the upper jejunum. One milligram of B_{12} was given parenterally at 0, 12, and 24 hours. The balloon was released at 12 hours (material was retained in duodenum and proximal jejunum by x-ray). Twenty-one hundredths per cent $B_{12}Co^{60}$ was excreted during the first, 0.15 per cent during the second, and 0.27 per cent during the third 12-hour periods. The same mixture orally with the same injections gave 3.9 per cent during the first, 8.2 per cent during the second, and 2.6 per cent during the third 12-hour periods. Barium was in the terminal ileum and colon by 12 hours. Thus, *vitamin B_{12} cannot be absorbed by the upper small bowel, and, as a matter of fact, IFC activity is destroyed by too long a sojurn there.*

Cellular enzymes undoubtedly act (with IFC help) in the transport of B_{12} from gut lumen into circulation. Carbonic anhydrase might conceivably be involved. Two hundred fifty milligrams acetazolamide (Diamox) was given every 8 hours for 3 days to 2 patients with pernicious anemia. Schilling tests using 1 unit IFC in one patient and 0.5 unit in the other gave 13.8 per cent and 10.5 per cent excretions, respectively, before, and 16.7 per cent and 10.5 per cent on the third day of acetazolamide administration. Thus, *carbonic anhydrase is not implicated.*

9. ELECTROPHORETIC STUDIES OF THE SERUM PROTEINS IN ACTIVE ULCERATIVE COLITIS

RICHARD O. BICKS, M.D. (BY INVITATION), JOSEPH B. KIRSNER, M.D., AND WALTER L. PALMER, M.D., CHICAGO, ILL.

The serum proteins were studied serially by filter paper electrophoresis in 23 patients with active ulcerative colitis. For comparison, normal individuals and patients with rheumatoid arthritis, systemic lupus erythematosus, cirrhosis of the liver, multiple myeloma, nontropical sprue, regional ileitis, and healed ulcerative colitis were studied similarly.

The protein partition in active ulcerative colitis followed two characteristic patterns; most commonly, hypoalbuminemia and elevations of alpha-2 and

gamma globulin (15 patients); less frequently, hypoalbuminemia and elevation of alpha-2 globulin only (8 patients). The first pattern has been described in collagen diseases and resembles that observed in patients with rheumatoid arthritis, scleroderma, and systemic lupus.

The effect of standard medical treatment, often supplemented with corticotropin or adrenal steroids, upon the electrophoretic patterns also was studied serially. The clinical response in those patients with an elevated gamma globulin was prompt; the electrophoretic response was delayed but eventually the pattern became normal. In two patients "T" globulins were seen but these disappeared with steroid therapy; the clinical response to steroids in these cases was dramatic. In the second group, i.e., hypoalbuminemia with elevated alpha-2 only, steroids were considerably less effective; large amounts were required to achieve any clinical response and complications of such therapy were more frequent.

Steroids do not influence antibody formation but they interfere with antigen-antibody reactions of the hypersensitivity type. The response to steroids in ulcerative colitis and the accompanying return of the abnormal protein patterns toward normal are in accord with the concept of a hypersensitivity mechanism in active ulcerative colitis. Electrophoretic patterns of the serum proteins apparently help to select patients who will respond to steroids and thus provide a more rational basis for their use. The "T" globulins found in two patients suggest that an immunologic mechanism also may be involved in the pathogenesis of the disease.

10. THE GENETIC MECHANISM OF IDIOPATHIC HYPERLIPEMIA

JOSEPH D. BOGGS, M.D., DAVID YI-YUNG HSIA, M.D., ROLAND F. MAIS, M.D., AND JOHN A. BIGLER, M.D., CHICAGO, ILL.

(INTRODUCED BY RALPH DOLKART, M.D.)

The propositus, a 9-year-old girl of Puerto Rican origin, was first seen because of multiple xanthomata. Subsequently, she developed precordial pain, and died suddenly from cardiac complications. On post-mortem examination, there was atherosclerosis involving the endocardium and the medium- and large-sized arteries, including the blood vessels of the brain. Death was due to occlusion of the coronary vessels and infarction of the myocardium.

Serum samples were obtained from all members of the immediate family on two separate occasions. These were analyzed for total lipids, phospholipids, cholesterol, and cholesterol esters. In addition, the serum proteins and lipoproteins were separated by paper electrophoresis. The following changes were found: (1) In addition to the propositus, two asymptomatic siblings were found to have all of the characteristic changes of idiopathic hyperlipemia. (2) Both parents and two additional siblings showed elevations of total and esterified cholesterols. (3) The three affected children showed a virtual absence of the alpha liproproteins, suggesting that the basic defect in the disease process lies in an inability to convert beta liproproteins to alpha lipoproteins. This could possibly occur because of a genetically determined enzyme deficiency. (4) A bright blue hue is present over the beta lipoprotein in all three affected children. (5) An abnormal fluorescent, narrow band is seen over the beta globulin area in two affected children and under the gamma globulin area in the third child.

It is generally agreed that a recessive mode of inheritance is suggested if the following criteria are present: (1) The presence of the condition in siblings, but not among ancestral or collateral relations. This has been shown in the

present pedigree. (2) If the condition is present in monozygotic, but not in dizygotic twins. This could not be tested in the present family. (3) If there is a history of consanguinity in the family. The parents of this family are first cousins. (4) If the heterozygous carriers of the abnormal gene are detectable by means of laboratory tests. This has been shown in this family.

This indicates that idiopathic hyperlipemia should be classified among the "inborn errors of metabolism" and the condition appears to be transmitted by a recessive gene.

11. ACID MUCOPOLYSACCHARIDES IN PLEURAL, PERICARDIAL, AND ASCITIC FLUIDS

ALFRED JAY BOLLET, M.D., MARIA W. SERAYDARIAN, PH.D. (BY INVITATION), AND WILLIAM F. SIMPSON, M.S. (BY INVITATION), DETROIT, MICH.

Polyserositis is a frequent occurrence in systemic connective tissue diseases. Since connective tissue is characterized by a high content of acid mucopolysaccharides, it was decided to determine whether these substances are present in effusions in serous cavities. Pleural, pericardial, and ascitic fluid from 14 patients was studied, and found to contain acid mucopolysaccharide. The concentration ranged from 44 to 1,470 μg per cent, measured as uronic acid by the carbazole method. The highest levels were observed in a pleural and a pericardial fluid from two patients with systemic lupus erythematosus, and ascitic fluid from a patient with carcinomatosis. In general, the lowest levels were found in fluid from patients with congestive heart failure and cirrhosis of the liver. Following incubation with testicular hyaluronidase, acid mucopolysaccharide could no longer be detected by the methods used.

Chromatographic studies of the acid mucopolysaccharide isolated from several fluids, using a propanol-phosphate buffer system, revealed the presence of two components staining metachromatically. One spot had the mobility of chondroitin sulfate and heparin, which are not separated by this solvent. The second spot remained at the origin, as did samples of hyaluronic acid. All the fluid examined in this fashion showed the same two components. Plasma samples obtained from these patients as well as many other individuals have revealed the presence of components with the chromatographic mobility of chondroitin sulfate and heparin, but none resembling hyaluronic acid. In addition, the hexosamine isolated from acid mucopolysaccharide of ascitic fluid was found to consist of 58 per cent glucosamine and 42 per cent galactosamine, whereas that from plasma contained mainly galactosamine. Hyaluronic acid has been reported to be present in relatively high concentration in fluid from a patient with mesothelioma of the pleura.

It is concluded from this data that at least one acid mucopolysaccharide present in pleural, pericardia, and ascitic fluid is derived from the lining membrane of these serous cavities. Chromatographic studies and the relatively high glucosamine content suggest that it is hyaluronic acid.

12. THE USE OF A SALINE CONDUCTIVITY METHOD FOR THE DETERMINATION OF CARDIAC SHUNTS BY THE INDICATOR-DILUTION TECHNIQUE

RICHARD W. BOOTH, M.D. (BY INVITATION), JOSEPH M. RYAN, M.D., AND RICHARD S. GOODWIN, M.S. (BY INVITATION), COLUMBUS, OHIO

The indicator-dilution technique is an accepted method for the determination of the site and type of cardiovascular shunting. The most commonly

employed indicator has been Evans blue with the dye curves being recorded photometrically.

A new method has been developed for this technique, utilizing the principle of a saline induced increase in blood electrical conductivity. The indicator, 5 per cent saline, is injected into different sites in the right heart during cardiac catheterization and the dilution curves are obtained from systemic arterial blood drawn through the conductivity cell, which is a hollow plastic cylinder with two imbedded platinum electrodes. The remaining instrumentation consists of a simple modification of any commonly used carrier amplifier plus a standard recorder.

This method has been used successfully in 21 patients with congenital heart disease, 12 of whom had shunts. Its validity has been established by: (1) simultaneous comparison with dye curves, (2) catheterization data, (3) cardiac surgery, and (4) autopsy. Representative curves from 4 patients will be shown as follows: (1) a pulmonic stenosis without shunting, (2) an Eisenmenger complex with a right-to-left shunt where simultaneous dye and conductivity curves showed identical contours and time bases, (3) a ventricular septal defect where four curves, one from the pulmonary artery and three from the right ventricle showed bidirectional but predominately left-to-right shunting, and (4) a tetralogy of Fallot with a curve from the right ventricle showing a right-to-left shunt.

The method retains the desirable feature of the Evans blue technique in that the curve is immediately available. It possesses, in addition, the following advantages: (1) determinations can be made as frequently as desired because there is no appreciable accumulation of indicator resulting in increased background in the blood; (2) saline does not discolor the patient; (3) saline does not interfere with cuvette oximetry as Evans blue does; therefore, curves can be obtained at any time during cardiac catheterization; and (4) most of the necessary instrumentation is accomplished by the simple and inexpensive modification of commonly used amplifier-recording systems.

13. THE FUNCTIONAL CAPACITY OF PERSISTING NEPHRONS IN EXPERIMENTALLY INDUCED CHRONIC BRIGHT'S DISEASE

NEAL S. BRICKER, M.D., HERBERT LUBOWITZ (BY INVITATION), RICHARD R. DEWEY, M.D. (BY INVITATION), JAMES S. STOKES, M.D. (BY INVITATION), AND DONALD Y. GOLDMAN (BY INVITATION), ST. LOUIS, MO.

The contention is widely held that the kidney with chronic Bright's disease is no longer a "true" kidney in that its constituent nephrons are disorganized functionally as well as morphologically. Using a method previously described, permanent unilateral renal disease has been induced in dogs. All damaged nephrons are thereby confined to one kidney. Comparison of the simultaneous functions of both kidneys under a variety of experimental conditions has allowed examination of the integrity of the persisting nephrons in the altered kidney.

Within two to four months after the experimental procedure (perfusion of the anoxic kidney with 2,6-dimethylaminopurine-3-amino-d-ribose, diverting renal venous blood to prevent recirculation), the involved kidneys become contracted, often to one-fourth their original size. Clinically, the animals remain healthy and develop neither hypertension nor progressive anemia. Histologically, the diseased kidneys show sclerosis of glomeruli, dilation, and atrophy of tubules and fibrosis. Intact kidneys have remained normal.

Functionally (GFR, ERPF, Tm_{PAH}, and $Tm_{glucose}$), the diseased kidneys show marked and persistent impairment, indistinguishable from contracted

kidneys of human Bright's disease. After an initial increase, the normal kidneys remain at approximately control levels of function.

Consistency of clearance values (after stabilization) for prolonged periods plus comparability of values for C_{Cr}/C_{PAH}, C_{Cr}/Tm_{PAH} and $C_{Cr}/Tm_{glucose}$ between the two kidneys, support the validity of clearance techniques in studying the diseased kidneys. These same data attest to the integrity of the persisting nephrons in the contracted kidney. Thus the volume of glomerular filtrate per unit of effective renal plasma flow and glomerulotubular balance (C_{Cr}/Tm_{PAH} and $C_{Cr}/Tm_{glucose}$) have remained equal bilaterally although GT balance changed in both kidneys after induction of unilateral lesions such that GFR per nephron increased. This suggests that functioning nephrons in both normal and diseased kidneys adapt to a decrease in total nephron population by increasing GFR. The hypothesis that this is the major adaptive change occurring in the contracted kidney is currently being evaluated.

It is concluded that nephrons in the contracted kidney are reduced in number but possess normal functional characteristics.

14. THE COLLATERAL CIRCULATION IN CORONARY HEART DISEASE: "BENIGN" or "MALIGNANT"?

BERNARD L. BROFMAN, M.D., CLEVELAND, OHIO

(INTRODUCED BY HARRY GOLDBLATT, M.D.)

In view of the absence of any obligate correlation between the location and extent of the occlusive process in the coronary arteries and the clinical-pathologic consequences thereof, our comprehension of fundamental pathophysiologic relationships in this disease has particularly suffered from a natural (and profound) reluctance (for fear of untoward reactions) to perform such vital studies in patients with clinical coronary heart disease. Consequently, a priori reasoning and empirical concepts abound.

This presentation is based upon a unique opportunity for *direct* hemodynamic and metabolic observations on the heart in more than 300 patients with *clinical* evidence of coronary heart disease operated upon by Dr. Claude Beck. A large control group was provided by over 500 patients undergoing thoracic surgery for other than coronary heart disease. In these studies, direct insertion of a catheter into the coronary sinus (or a suitable coronary vein) permitted serial determinations of the myocardial extraction coefficient ($\frac{A\text{-}V}{A}$) for O_2 and various other substrates. From these studies has emerged a more or less unexpected observation: Generally, there was no definite *increase* in oxygen extraction, as would be anticipated a priori in patients with coronary heart disease; on the contrary, particularly in patients with extensive myocardial scarring, there was a significant *decrease* (*increased* coronary venous O_2 content). This apparently paradoxical situation is further confirmed by experimental, clinical, and anatomic observations, and should stimulate a critical reappraisal of many empirical and old "established" concepts in coronary heart disease. With adequate collaterals to provide uniform distribution via a final common pathway for the ubiquitous red blood cell, the integrity of the myocardium may be maintained almost indefinitely despite multiple complete coronary artery occlusions. However achieved, intercoronary anastomoses *in advance* of the occlusive process are truly beneficial; the extensive collaterals resulting from coronary occlusion and myocardial infarction are not necessarily

so, and may actually be deleterious in that they encourage perfusion of a low-resistance scar. This preferential flow then deprives adjacent viable myocardium of critically needed blood.

15. LEVELS OF ANTIBODY AGAINST DENVER A′-1957 AND ASIAN-1957 INFLUENZA VIRUSES IN HUMAN AND ANIMAL SERA IN AN AREA NOT YET INVOLVED IN AN EPIDEMIC OUTBREAK

G. O. Broun, M.D., Rose Rita Schmidt, M.S. (By Invitation), and Sr. M. Laureen Harris, S.S.M., B.S. (By Invitation), St. Louis, Mo.

Observation of the incidence of influenza in the area of St. Louis, Mo., in the past 14 years by clinical and laboratory studies permits an evaluation of changes in antibody levels occurring in the population in recent months. Studies of paired sera, culture of throat washings, examination of sera of infants, and of serum pools collected according to date of birth give evidence concerning the prevalence of recently isolated strains of influenza virus in this area.

There is evidence that an A′ strain isolated in Colorado by Dr. Meikeljohn in January, 1957, in all probability reached the St. Louis area within a month after its isolation but without causing a clinically recognized outbreak. Only a few cases showed antibody rises against this virus with accompanying clinical symptoms of acute influenza. Evidence for the presence of the virus in the area is found by examination of infant sera collected consecutively by months, during the winter and spring. This virus shows some cross reactions with A′ strains isolated in St. Louis in 1953. Individuals born between 1895 and 1905 show the highest levels of "natural" antibodies against the Denver virus. Animal sera collected in this area showed antibodies against this strain as soon as and perhaps even earlier than human sera collected simultaneously.

Antihemagglutinins against the Asian strain which had been passed only in eggs were found in but a single 80-year-old individual in the St. Louis area up to Sept. 1, 1957. He had no evidence of clinical influenza. The strain passed through mice has been much more reactive. Highest antibody titers have been found in those over 60 years of age. Examination of throat washings and paired sera from "influenza-like" illness in this area up to Sept. 1, 1957, have shown no definite evidence that the infection has spread to this vicinity. Animal sera collected up to this date show no antibodies against the Asian strain of virus.

16. SUGGESTED REVISION OF NOMENCLATURE FOR INTRAVENTRICULAR CONDUCTION "DEFECTS"

J. Marion Bryant, M.D., S. I. Said, M.D. (By Invitation), H. B. Youn, M.D. (By Invitation), and Thomas M. Cook, M.D. (By Invitation), New York, N. Y.

Practically all types of so-called defects of intraventricular conduction have been demonstrated by the electrocardiograms of healthy young subjects. The conventional pathologic connotation of the term "bundle branch block" makes a more objective and purely descriptive nomenclature desirable to aid in breaking with the concept that electrocardiographic abnormalities of themselves are diseases. Such a change would serve as a step toward reducing the incidence of electrocardiographic-induced iatrogenic heart disease.

A comparative study of 200 healthy young subjects and 100 older cardiac patients serves as the basis for suggesting a revision of electrocardiographic nomenclature. Based on the form of the QRS complex, "normal intraventricular conduction" (classic concept) occurred in 2 per cent of healthy subjects and 50 per cent of cardiac patients, "right bundle branch block" in 95 per cent of healthy subjects and 22 per cent of cardiac patients, "left bundle branch block" in 13 per cent of healthy subjects and 22 per cent of cardiac patients, and "bilateral bundle branch block" in 10 per cent of healthy subjects and 3 per cent of cardiac patients. The QRS interval is of little value in assessing the presence or degree of intraventricular conduction "defects," and was found to be shorter, on the average, in cardiac patients than in healthy subjects.

A new nomenclature, based on the form of the QRS complex in right-sided unipolar leads and modified by the value of the QRS interval, is suggested. The terminology of the American Heart Association is used. Thus, "RS-type" for the classic type of "normal intraventricular conduction," "RSR-type" for "right bundle branch block," "QS-type" for "left bundle branch block," and "QRSR-type" for "bilateral bundle branch block."

17. REFLECTIONS OF THE AGING PROCESS IN MAN IN HIS SPATIAL VECTORCARDIOGRAM

G. E. Burch, M.D., and Lawrence Golden, M.D. (By Invitation),
New Orleans, La.

With aging, gradual and subtle progressive changes develop in the tissues, including the cardiovascular system. It was thought that such progressive changes with aging may be reflected in the spatial vectorcardiogram (sVCG), which records electrical events associated with the heartbeat in greater detail than the conventionally recorded ECG.

The sVCG was recorded in 224 normal subjects varying in age from 20 to 72 years. A close similarity was noted for all age groups in magnitude and spatial orientation of the maximal mean instantaneous vectors of the QRS and T sÊ-loops with horizontal orientation being greater in the older people. The QRS sÊ-loop progressively and subtly developed "distortions" which rose from an incidence of 22 per cent in the subjects of the third decade of life to 88 per cent in those of the seventh decade of life.

The significance and mechanisms of these changes remain unknown, but it would appear that they reflect in large part progressive alterations in the order of depolarization with aging, a newly noted expression of senescence. A more sensitive galvanometer and higher paper speed would record electrocardiographically some of the alterations. Such myocardial electrical alterations with age may prove to be clinically useful now that some may be objectively recorded.

18. A SIMPLE, RAPID, AND ACCURATE T-1824 EXTRACTION METHOD SUITABLE FOR ROUTINE MEASUREMENTS OF BLOOD VOLUME

T. J. Campbell, B.S. (By Invitation), B. Frohman, B.A. (By Invitation), and
E. B. Reeve, M.D., Denver, Colo.

Methods for measuring blood volume have required special equipment or special care, and usually both, and have not been suitable for use in the average hospital laboratory. Of the methods available the dye methods come nearest to being satisfactory, but direct measurement of the optical density of dyed

plasma requires skill and is rendered inaccurate by hemolysis and varying opalescence of the samples, and none of the described extraction methods are without their difficulties. T. H. Allen's method (1951, 1953), in which T-1824 is displaced from albumin by detergent, adsorbed to cellulose, and eluted into acetone-water is the best of the latter, but requires 3 hours for the extraction of the dye, too long a period for its use in the management of the emergency patient. We have modified Allen's method as follows. One to four milliliters of dyed plasma is treated with detergent in sodium phosphate solution. The mixture is passed through a wood pulp column (Solka Floc S.W 40A) and the adsorbed dye is freed from protein and pigments by a wash of the phosphate solution. The dye is then eluted with acetone-water, buffered, and the optical density is read in a Coleman Junior or other simple spectrophotometer. A standard prepared in the patient's blank plasma is put through the same process. Ninety-seven ± 2 per cent of the dye is extracted in 20 minutes at room temperature, the optical density of the plasma blanks is zero, and hemolysis, bilirubinemia, and opalescence do not interfere. Measurements made by this method show satisfactory agreement with those made by the direct method. The method seems applicable to all patients. Since results can be obtained 60 to 70 minutes after dye injection, it should prove particularly useful for the management of patients suffering from hemorrhage.

19. CORRECTION OF DEFECTS IN CLOTTING ACCELERATOR FACTORS BY ADMINISTRATION OF A NEW SULFHYDRYL-SUBSTITUTED METHYLNAPHTHOQUINONE, VITAMIN K-S(II)

JOHN R. CARTER, M.D. (BY INVITATION), AND E. D. WARNER, M.D.,
IOWA CITY, IOWA

Two patients, critically ill with severe hemorrhagic disorders caused by a deficiency of accelerator factor(s) of blood coagulation, will be presented. One has been studied intensively for over six years. The deficiencies were corrected and the bleeding was controlled promptly and completely by the oral and/or intravenous administration of S-(2-methyl-1, 4-naphthoquinonyl-3)-β-mercapto-propionic acid, designated vitamin K-S(II). Banked and fresh blood in both patients and massive doses of vitamin K and K_1 in one patient were ineffective.

The design and preparation of vitamin K-S(II) was based upon experimental studies which showed that the accelerator activity in blood coagulation is influenced strikingly by the concomitant administration of DL-methionine and vitamin K. Because of the synergistic effect of methionine and vitamin K, vitamin K-S(II) was designed to incorporate both sulfur and propionic acid, a carbon chain product of methionine degradation, into the vitamin K molecule in the 3-position. Experimental and clinical studies in normal dogs and rats, cholecystnephrostomized dogs on normal, protein, or methionine-free diets, partially hepatectomized rats, and in patients have shown the vitamin K-S(II) to be fully as effective as the methionine plus vitamin K.

20. SHORTENED SURVIVAL OF INCOMPATIBLE RED CELLS IN PATIENTS WITH "AGAMMAGLOBULINEMIA": A SENSITIVE TEST FOR THE PRESENCE OF TRACES OF ISOANTIBODY

HUGH CHAPLIN, JR., M.D., ST. LOUIS, MO.

Patients with agammaglobulinemia have been characterized as "universal recipients" because of the absence of anti-A and anti-B isoagglutinins from

their sera. The present report describes the survival of ABO-*incompatible* red cells in the circulations of 4 adult patients whose sera showed an absence of gamma globulin on paper electrophoresis.

Although routine tests for anti-A and anti-B isoagglutinins were negative, more sensitive tests revealed very weak isoagglutinins in 3 of the 4 patients. Ten milliliters of Cr^{51}-tagged incompatible red cells were injected intravenously over a 2-minute interval. No untoward symptoms or signs were observed. Red cell survivals were shortened in all 4 patients, with Cr^{51} half-times from less than 10 minutes to as long as 9 days (Normal: 30 ± 3 days).

Although the in vitro tests for antibody activity revealed no qualitative differences among the sera from the 3 patients with detectable isoagglutinins, surface scintillation counting over the hepatic and splenic areas suggested two different mechanisms for red cell removal. One mechanism (in two patients) entailed nearly equal participation by both liver and spleen; the other (in one patient) was almost exclusively a function of the spleen.

The sensitivity of the above-described in vivo studies for the detection of minute amounts of circulating antibody was illustrated by a second study performed on one of the patients. Ten milliliters of Cr^{51}-tagged incompatible blood was injected and the rapid disappearance of the red cells (less than 10 per cent survival after 10 minutes) confirmed as described previously. One hour later, a pint of Cr^{51}-tagged blood from the same donor was transfused; 90 per cent of the incompatible cells were present in the recipient's circulation at the conclusion of the transfusion. There was no measurable destruction of the surviving red cells during the succeeding 24 hours, and their survival was followed during the subsequent 6 weeks by both the Ashby and the Cr^{51} methods, along with surface scintillation counting over the liver and spleen. The results provide an interesting comparison of the destruction of red cells under conditions of antibody excess compared to antibody insufficiency.

21. PEREGRINATING PROBLEM PATIENTS: "MUNCHAUSEN'S SYNDROME"

JOHN S. CHAPMAN, M.D., IOWA CITY, IOWA

(INTRODUCED BY ROBERT C. HARDIN, M.D.)

Not before reported in this country is an instance of "Munchausen's syndrome," so named by Richard Asher of England. The term was suggested by similarities between the bizarre symptoms and behavior exhibited by certain patients and the tales of wild adventure and personal achievement told by the fabulous Baron Munchausen. Patients with Munchausen's syndrome travel widely among hospitals feigning symptoms and producing factitious lesions which require emergency care or extensive diagnostic investigations. One such patient was admitted to the University of Iowa Hospitals, Iowa City, Iowa, August 18, 1954, because of hemoptysis and thrombophlebitis.

In the following days there unfolded a series of remarkable events starting with plausible symptoms and progressing through inappropriate behavior to uncontrollable violence and fantastic self-inflected wounds. Disposition was finally made to a state mental hospital from which he subsequently fled.

By correspondence, it was learned that this was a repetitive pattern which the patient had been following for several years, and which continued after leaving Iowa. In the months following he was admitted to at least thirteen hospitals from California to New York with similar occurrences in all. The admitting diagnosis has always been thrombophlebitis and pulmonary embolus,

while his discharge diagnoses have included a variety of psychiatric and other disorders. His method of producing hemoptysis has not been determined despite extensive examinations. In the past he has had many surgical procedures including inferior vena cava ligation, which partly accounts for the continuing signs of thrombophlebitis. He always concocts a farfetched story to explain the abdominal scar, and refuses to cooperate in treatment.

This patient is representative of one of the subgroups of the syndrome which has been called the bleeding type or "hemorrhagica histrionica." He also has features of the abdominal type or "laparotomophilia migrans."

These patients are so nearly alike in their behavior that it seems useful to classify them as a group, using the descriptive term "Munchausen syndrome" so long as the exact psychiatric diagnosis remains unknown. It is thought that many such patients exist, although hitherto not recognized in this country.

22. PULMONARY CAPILLARY FLOW-PRESSURE RELATIONSHIPS

BERNARD L. CHARMS, M.D., AND BERNARD L. BROFMAN, M.D., CLEVELAND, OHIO

(INTRODUCED BY ERWIN LEVIN, M.D.)

Studies have been done employing a triple lumen cardiac catheter with an inflatable cuff allowing pressure measurements and blood aspiration proximal and distal to the point of occlusion. "Wedge" pressures and samples were also obtained by standard techniques. Since wedged and distal pressures were identical both in level and configuration, the latter were used when standard "P. cap." was unobtainable due to pulmonary hypertension.

Pressures distal to occlusion were normal in patients with no disease or those with localized pulmonary disease and elevated with mitral valve or left ventricular involvement. Diffuse pulmonary disease produced marked variations in measured distal or "P. cap." pressures coincident with the respiratory cycle reaching high levels in the expiratory phase. Aspiration of wedged or distal blood samples was easily accomplished in normal patients or those with heart disease but difficult or impossible in many with chronic pulmonary disease. When obtainable, this blood was more highly oxygenated than arterial blood having been exposed longer to alveolar air and, therefore, more closely equilibrated. Increase in pulmonary artery pressure can be readily observed with exercise, however, simultaneous measurement of distal pressure showed a proportional increase without any presumed rise in left atrial pressure.

It is concluded that when blood flow is arrested in portions of the pulmonary vascular bed, the static "lacunar" pressure beyond the occlusion is closely related to left atrial pressure particularly when elevated. Under circumstances of increased intrapulmonic pressure, "lacunar" pressures reflect this change rather than left atrial. It is quite possible that increased intrapulmonic pressure may collapse pulmonary capillaries if it exceeds left atrial pressure causing what might be termed a "retrograde block."

Evidence is presented that ordinary "wedge" pressures, particularly in patients with primary pulmonary disease, are not reliable indicators of true pulmonary capillary pressure and may lead to completely invalid conclusions regarding left atrial hemodynamics. These studies support the contention that regulation of pulmonary blood flow is extravascular unlike the intrinsic arteriolar control of the periphery.

23. CLINICAL OBSERVATIONS ON THE USE OF NOVOBIOCIN IN PENICILLIN RESISTANT STAPHYLOCOCCAL SEPTICEMIA

J. M. Colville, M.D., H. H. Gale, M.D., F. Cox, Jr., M.D., and E. L. Quinn, M.D., Detroit, Mich.

(Introduced by R. W. Smith, M.D.)

The seriousness of staphylococcal septicemia is indicated by a continuing mortality rate of 50 to 80 per cent despite modern antimicrobial therapy. Thus, new antimicrobial agents demonstrating antistaphylococcal activity are worthy of careful consideration and study.

Novobiocin, an antibiotic agent derived from *Streptomyces niveus* and demonstrating both in vitro and in vivo antistaphylococcal activity, became available for investigational study approximately two years ago. Subsequent investigations in our own and other laboratories indicated that this agent might prove useful in the treatment of staphylococcal sepsis due to penicillin-resistant organisms.

During the past 20 months, 7 patients with staphylococcal sepsis have been treated with novobiocin. The following significant features were common to all cases and preceded the onset of staphylococcal sepsis in all cases: an underlying primary disease process, hospitalization, surgical intervention, and previous exposure to various antimicrobial agents. In each instance the isolated strains of staphylococci were pigmented, hemolytic, and coagulase positive.

Five of the 7 patients responded satisfactorily to treatment with novobiocin. Of the remaining 2 cases, 1 developed severe thrombocytopenia purpura, a previously unreported toxic reaction, on the ninth day of therapy after receiving 18 Gm. of novobiocin. In the other patient, emergence of a novobiocin resistant strain of staphylococcus occurred after 7 days of therapy.

24. REVERSAL OF THE LESIONS OF THE GENERALIZED SHWARTZMAN PHENOMENON BY TREATMENT OF RABBITS WITH STREPTOKINASE

Richard M. Condie (By Invitation), C. Y. Hong, M.D. (By Invitation), and Robert A. Good, M.D., Minneapolis, Minn.

The generalized Shwartzman reaction is associated with deposition of homogeneous, eosinophilic material with staining characteristics of fibrinoid. Deposits occur within the glomerular capillaries, splenic sinusoids, hepatic veins, and pulmonary arteries, as well as within cardiac valves and beneath endothelium of coronary arteries. In the developing renal lesion, this fibrinoid material becomes visible at the inner surface of the glomerular capillaries between 2 and 4 hours following the second intravenous injection of endotoxin and is primary to the bilateral renal cortical necrosis which develops later. Prevention of the Shwartzman reaction with heparin and other anticoagulants effectively inhibits development of these fibrinoid lesions, but only when administered at the time of, or prior to, the provoking injection of endotoxin. Heparin administered following the second injection of endotoxin appeared to enhance the lesions.

In an effort to correct deposition of fibrinoid material and prevent consequent hemorrhagic necrosis, streptokinase, and streptodornase mixtures were

given intravenously to rabbits at a time when it was known that fibrinoid deposits had already been formed. This treatment appeared to reverse the fibrinoid deposition and prevented development of renal necrosis. It was found that this effect is due to streptokinase and will prevent completely the generalized Shwartzman reaction when streptokinase is administered up to 4 hours following the second intravenous injection of endotoxin. Quantities of streptokinase effective in preventing the Shwartzman lesions were found to have no effect on the febrile response development of leukopenia and leukocytosis, inhibition, leukocytic migration, decrease in platelet count, production of heparin precipitable protein, or increase in fibrinogen levels which normally accompany injection of endotoxins. Administration of streptokinase resulted in immediate activation of fibrinolytic capacity of the blood which persisted for periods of more than 3 hours.

Similar results were obtained with the local Shwartzman reaction and the visceral vascular reaction produced by a single intravenous injection of endotoxin together with polyethanol sulfonate.

We conclude from these studies that deposits of fibrinoid material which occur in the course of the generalized Shwartzman reaction are reversible by treatment with streptokinase and that such treatment prevents the progress of the lesions to hemorrhagic necrosis.

25. CHYLOMICRON-LIPOPROTEIN DISTRIBUTION AND THE DYNAMICS OF LIPID TRANSPORT

David G. Cornwell, Ph.D. (By Invitation), Fred A. Kruger, M.D. (By Invitation), George J. Hamwi, M.D., and J. B. Brown, Ph.D. (By Invitation), Columbus, Ohio

Although lipids are absorbed from the intestine and transported in serum by a well-defined chylomicron-lipoprotein system, the origin of specific components in this system and their metabolic interrelationships are not understood. Metabolic transformations between different lipoproteins have not been investigated by the usual tracer techniques, since labeled lipids (cholesterol and phospholipid) in one lipoprotein component of the system were shown to exchange, in vitro, with lipids in the other lipoprotein components. In the present investigation, triolein tagged with I^{131} was found suitable to study chylomicron and lipoprotein transformations since the labeled triolein did not exchange, in vitro, between different lipoprotein classes.

The chylomicra and lipoproteins in human serum were partitioned by ultracentrifugal flotation in a density gradient (Oncley and associates). Quantitative lipoprotein distributions were then estimated from cholesterol and phospholipid analyses of each lipoprotein fraction. Three characteristic lipoprotein distribution patterns were observed: (1) Normal; (2) Hyperlipemia in S_f 10-400 lipoproteins; and (3) Hyperlipemia in S_f 3-9 lipoproteins. In patients with a clinical diagnosis of idiopathic hyperlipemia, xanthoma tuberosum, the nephrotic syndrome, and the Kimmelstiel-Wilson syndrome, an elevation in S_f 10-400 lipoproteins occurred. An elevation in S_f 3-9 lipoproteins was found in some patients with a clinical diagnosis of diabetes mellitus, atherosclerosis, and in all patients with myxedema.

In normal individuals, iodinated triolein was absorbed and transported primarily by the chylomicra. The iodinated triglyceride reached its maximum concentration in serum about 4 hours after administration and was cleared from the serum in 24 hours. Labeled triglyceride was not found in either

S_f 3-9 or high density lipoproteins. Individuals with an S_f 3-9 hyperlipemia absorbed and transported triolein in the same manner as normal individuals. Individuals with an S_f 10-400 hyperlipemia had a distinctly altered pattern. Labeled triolein was carried primarily in the S_f 10-400 lipoprotein fraction. Furthermore, the triolein was cleared from the serum at a slower rate.

These results may be interpreted in at least two ways: (1) S_f 10-400 lipoproteins are derived from chylomicra. S_f 10-400 hyperlipemia results from a decrease in the rate of clearing for this lipoprotein class, and (2) chylomicra and S_f 10-400 lipoproteins are synthesized independently. S_f 10-400 hyperlipemia results from both increased synthesis and decreased clearing in this lipoprotein class.

26. CONTROLLED STUDY OF A POLYVALENT ADENOVIRUS INFLUENZA VACCINE

F. Cox, Jr., M.D., E. A. Timm, Ph.D., E. L. Quinn, M.D., J. M. Colville, M.D., and I. William McLean, M.D., Detroit, Mich.

(Introduced by John E. Rebuck, M.D.)

A controlled study concerning antibody response and tissue tolerance to polyvalent adenovirus influenza vaccine was undertaken in November, 1956.

Three groups of approximately 37 individuals each were studied. Group I received fluid vaccine containing adenovirus types 3, 4, and 7, Influenza type A (PR8, PR301, and Swine), and Influenza B (Great Lakes and Lee). Group II received a vaccine containing the same antigens which had been absorbed onto aluminum phosphate. Group III was given a placebo consisting of balanced salt solution. A single booster was administered 16 weeks after primary vaccination. Control prevaccination sera were drawn from all subjects at the onset of the program and thereafter at 2, 16 (prebooster), and 18 weeks. Additional sera were obtained from one-third of the subjects in each group at 6, 10, and 14 weeks. All sera were tested in tissue culture for neutralizing antibodies against type 3, 4, and 7 adenovirus. Influenza hemagglutination inhibition tests were performed on both control and postvaccination sera.

The results showed that adenovirus antigen combined in an adenovirus influenza vaccine elicted a satisfactory antibody response. The magnitude of the change in antibody titer was similar to that observed by Bell and coworkers when adenovirus antigen alone was employed. Aluminum phosphate absorbed vaccine produced superior antibody response when compared to the fluid vaccine.

Following a booster dose of polyvalent vaccine, increase in adenovirus antibody titer was noted only if the prebooster titer was fairly low or if the subject showed no antibody at the time of the booster. These results also indicated that the antibody titer resulting from vaccination was quite stable over an 18-week period.

Influenza hemagglutination inhibition antibody tests yielded an average arithmetical mean fold increase titer for all the contained strains of influenza virus of 0.3 in the control group and 4.5 and 3.9 in the two groups receiving the vaccine.

No significant differences in the tissue tolerance were noted with the two types of vaccine.

27. A COMPARISON OF THE EFFECTS OF TOLBUTAMIDE AND INSULIN ON PERIPHERAL GLUCOSE UPTAKE IN MAN

JAMES W. CRAIG, M.D. (BY INVITATION), VALERIE J. MOLZAHN (BY INVITATION), WILLIAM R. DRUCKER, M.D. (BY INVITATION), MAX MILLER, M.D., AND HIRAM WOODWARD, JR. (BY INVITATION), CLEVELAND, OHIO

The mechanism of the hypoglycemic action of the sulfonylureas has not been explained satisfactorily. Several workers have compared the metabolic effects of insulin and the sulfonylureas. The results of these investigations have been conflicting, particularly in regard to the effect of the sulfonylureas on peripheral glucose uptake.

In the present study the direct effects of tolbutamide and insulin on glucose uptake in the forearm were compared by injecting the drugs into the brachial artery. Two tests were performed on each of 6 male patients; on one occasion tolbutamide was injected and at another time insulin was used in order to compare the two compounds in the same individual. Blood samples were obtained simultaneously through indwelling needles in the brachial artery and a major antecubital vein. Two-tenths or 0.4 Gm. of sodium tolbutamide (Orinase) or 0.01 unit per Kg. of glucagon-free insulin were injected into the brachial artery and blood samples were drawn at 5 or 10 minute intervals for the next hour. Insulin increased the A-V glucose difference in 5 of the 6 subjects; the mean difference ± s.d. was 0.3 ± 2.0 mg. per 100 ml. before injection and 6.4 ± 3.9 mg. per 100 ml. during the first 30 minutes after injection. Tolbutamide produced no definite increase in A-V glucose difference in any of the 6 subjects during the first 30 minutes after injection; the mean control value was 3.2 ± 2.3 mg. per 100 ml. and the mean value after tolbutamide was 1.3 ± 2.2 mg. per 100 ml. No direct stimulatory effect of tolbutamide on peripheral glucose uptake was demonstrated in contrast to the results with insulin.

In 2 out of 3 cases in which peripheral A-V glucose differences and peripheral arterial-hepatic venous glucose differences were determined simultaneously after injection of 2 Gm. of tolbutamide into a peripheral vein, the fall in arterial blood glucose was accompanied by a decreased splanchnic glucose output without an increase in peripheral A-V difference. The significance of these results in relation to current theories of the mechanism of action of tolbutamide will be discussed.

28. CHLOROTHIAZIDE: A PHYSIOLOGIC CONNECTING LINK

ARCHER P. CROSLEY, JR., M.D., AND ROBERT E. CULLEN, M.D. (BY INVITATION), MADISON, WIS.

The purpose of this paper is to demonstrate in acute and chronic studies in 16 patients with renal and/or cardiac disease the manner in which chlorothiazide, a nonmercurial diuretic, serves as a physiologic connecting link between the activity of carbonic anhydrase inhibitors and the mercurial diuretics. Thus, this agent which inhibits carbonic-anhydrase (C-A) in vitro is similar to acetazolamide, *p*-sulfamylbenzoic acid, and dichlorphenamide in its ability to produce significant reductions in glomerular filtration rate ($p = <0.01$) and renal blood flow ($p = <0.001$) while simultaneously causing a significant increase in urinary pH ($p = <0.01$) due to the increased excretion of sodium ($p = <0.001$) and potassium ($p = <0.01$). However, in contrast to C-A inhibitors, this compound, like the mercurial diuretics is capable of producing a significant

chloruresis ($p = <0.01$) and diuresis ($p = <0.001$). In a similar fashion, on chronic administration, a hypokalemic hypochloremic alkalosis may be observed rather than the hyperchloremic acidosis which may occur following the use of acetazolamide.

Therefore, these results (1) establish a physiologic connecting link between the C-A inhibitors and the mercurial diuretics, (2) demonstrate the effectiveness of chlorothiazide as a valuable adjunct in the treatment of edematous states, and (3) have physiologic significance when related to the renal mechanism involved in the excretion of water and electrolytes.

29. THE HEMODYNAMIC RESPONSE TO EXERCISE IN PULMONIC STENOSIS

EDWARD W. DENNIS, M.D., AND JAMES K. ALEXANDER, M.D. (BY INVITATION), HOUSTON, TEXAS

The hemodynamic response to exercise has been investigated in 5 women having isolated pulmonic stenosis without clinical evidence of congestive heart failure. Data were obtained during right heart catheterization utilizing a double lumen catheter to obtain simultaneous pressures from the right ventricle and pulmonary artery. The following parameters were examined at rest and during mild leg exercise: (1) cardiac output and stroke volume; (2) mean systolic pressure gradient across the stenotic ventricular infundibulum or pulmonary valve; (3) right ventricular end diastolic pressure; (4) pulmonary artery mean pressure.

There was a range of 20 to 150 mm. of mercury mean systolic pressure gradient between the right ventricle and pulmonary artery at rest with a tendency to rise as much as 15 mm. of mercury during exercise. In the two patients with the highest mean systolic pressure gradient, the resting cardiac output was reduced. However there was an increase in pulmonary blood flow during exercise in all patients ranging from a borderline normal value of 574 ml./100 ml. increase in oxygen consumption to a maximum of 1,710 ml. Mean pulmonary artery pressure rose in all cases during exercise as much as 10 mm. of mercury. Right ventricular end diastolic pressure rose to significantly elevated levels in all cases.

These data suggest that the observed normal increase in right ventricular output and the increase in mean systolic pressure gradient are achieved at the expense of significant elevation of right ventricular filling pressure in these patients.

30. TREATMENT OF HYPERTENSIVE DISEASE IN PREGNANCY

WILLIAM J. DIECKMANN, M.D., AND JOHN P. HARROD, M.D. (BY INVITATION), CHICAGO, ILL.

During the past 6 years approximately 1,200 patients have been treated for so-called "toxemia of pregnancy." Forty-five per cent of the patients had preeclampsia and 0.5 per cent had eclampsia but only two patients with these diagnoses received a hypotensive drug. In general, we believe the hypertension, if present, is compensatory and it is not treated unless the systolic blood pressure is 200 or higher with proportionate diastolic pressures.

Fifty-two per cent of the patients had hypertensive disease, the majority of some years' duration. Approximately 55 patients were hospitalized and

placed in an air-conditioned room with no other treatment than either the regular hospital diet or a low-sodium and low-potassium diet. A high percentage of the patients on the above regimen either became normotensive or the blood pressure dropped to such a degree that hypotensive drugs were no longer indicated; this emphasizes the importance of a control period.

Twenty patients who were seen sufficiently early in pregnancy and whose blood pressure persisted for 48 hours or longer despite the above regimen, were thoroughly studied and in each instance the offer was made of therapeutic abortion. These patients were all anxious to have the child if it could be done with reasonable safety. The patients were followed by one man as ambulatory patients and were hospitalized for 5 to 10 days every 4 to 6 weeks.

Seventy per cent of these patients left the hospital with a live baby, approximately the same per cent which Dieckmann has reported previously without the use of hypotensive drugs. To date, hypotensive drugs in the treatment of hypertensive disease in pregnancy have not decreased proteinuria and have not increased fetal survival.

31. EFFECT OF ORAL DIURETICS ON BLOOD SUGAR

Harold L. Dobson, M.D. (By Invitation), Harry S. Lipscomb, M.D. (By Invitation), and James A. Greene, M.D., Houston, Texas

Utilizing both the Somogyi-Nelson and glucose oxidase methods, the effects of acetazolamide (Diamox) and chlormaredrin (Neohydrin) on blood sugar have been investigated. Acetazolamide was found to act as a mild to moderate hypoglycemic agent in over one-half of the subjects tested. In some diabetics it appeared to be more effective than carbutamide. Certain patients develop untoward reactions when placed on this drug. In some, but not all, of these patients the symptoms are due to hypoglycemia. It is thought that this drug should be used with great caution in patients in whom mild hypoglycemia is known to be harmful.

Chlormaredrin reacts with the Somogyi-Nelson color reagent to give erroneously high blood sugar values. The error may be in magnitude of 400 to 500 mg. per cent. The effect may be found in vivo and in vitro. It is maximal within two hours after ingestion of the drug. This may lead to very confusing results when blood sugar studies are being performed on cardiac patients receiving chlormaredrin.

32. CARBOHYDRATE METABOLISM IN PATIENTS UNDERGOING SURGERY WITHOUT ANESTHESIA

William R. Drucker, M.D. (By Invitation), Charles A. Hubay, M.D. (By Invitation), William D. Holden, M.D. (By Invitation), Max Miller, M.D., James Craig, M.D. (By Invitation), Nancy Hofmann, A.B. (By Invitation), Hiram Woodward, A.B. (By Invitation), and David Weir, M.D., Cleveland, Ohio

Surgery with anesthesia, or ether anesthesia alone, will cause abnormalities in carbohydrate metabolism. Glucose, pyruvate, and lactate tolerance becomes altered, whereas fructose tolerance remains essentially unchanged following these stresses. It is possible that some of the reported metabolic changes found following surgery could be due to the anesthesia rather than the result of operative trauma.

The present study was designed to determine the effect of surgery without associated anesthesia on carbohydrate metabolism. Intravenous glucose, fructose, and lactate tolerance tests were performed on paraplegic patients operated for repair of decubitus ulcers. The surgical procedures could be carried out in these individuals, who had spinal cord injuries, without the use of anesthesia. Six patients received glucose and fructose tolerance tests on succeeding days before and after surgery. Six additional patients received lactate tolerance tests before, during, and following surgery. The levels of glucose, fructose, pyruvic acid, and lactic acid were determined periodically in venous blood.

The data obtained from these patients operated upon without anesthesia indicate that: (1) a marked abnormality occurs in postoperative glucose tolerance which persists for at least two days; (2) there is no abnormality in the postoperative tolerance for fructose; and (3) a decreased tolerance occurs for lactate during surgery and on the first postoperative day. Similar results were found with patients operated upon with anesthesia. In contrast, the abnormality in glucose tolerance which occurs during anesthesia without surgery disappears within twenty-four hours.

These results have been interpreted as indicating that surgical trauma causes alterations of intermediary metabolism which do not depend on associated anesthesia. Since the alterations of carbohydrate metabolism occur when surgery is carried out below the level of sensation in paraplegic patients, factors in addition to central nervous system stimulation are implicated in the etiology of these changes. It is postulated that the effect of surgical trauma without anesthesia is to alter specific enzymatic processes since the metabolism of glucose and lactate, but not fructose, are altered postoperatively.

33. COMPARATIVE DIAGNOSTIC VALUE OF URINARY COMPOSITION AND TRANSLUMBAR AORTOGRAPHY IN RENAL HYPERTENSION

HARRIET P. DUSTAN, M.D., EUGENE F. POUTASSE, M.D. (BY INVITATION), AND A. C. CORCORAN, M.D., CLEVELAND, OHIO

Occlusive renal artery disease, an important cause of hypertension, is best demonstrated currently by aortography which, with our technique, is a safe procedure. A recent report suggests that aortography is unnecessary in diagnosing a unilateral arterial lesion since the affected kidney is believed always to excrete a urine of smaller volume and lower sodium concentration. Analyses of ureteral urine are of no help in diagnosing bilateral lesions.

This report concerns a study of separated renal functions in hypertensive patients, some having renal arterial lesions. Urine was collected from each ureter during mannitol loading and Pitressin antidiuresis. Functions studied were glomerular filtration rate (GFR), urine flow (V), urine sodium concentration (U_{Na}), and the excretion fraction of the filtered sodium load (EF_{Na}).

The functions were bilaterally equal in 10 patients with essential hypertension. In 4 patients with (predominantly) unilateral pyelonephritis, GFR and V were less on the more affected side while U_{Na} and EF_{Na} were identical. In 4 with unilateral arterial lesions, GFR and V were depressed on the affected side; in 3 there were associated decreases of U_{Na} and EF_{Na}, but in the fourth these values were bilaterally equal. In two patients with bilateral arterial lesions, differences in GFR occurred between the two sides with corresponding V changes; U_{Na} and EF_{Na} were *higher* on the sides of lower GFR. In two with

unilateral arterial and contralateral parenchymal disease, GFR was lower on the sides with parenchymal disease; differences in U_{Na} were insignificant but EF_{Na} were *less* on the sides of the arterial lesions.

The data show a general association between GFR and V in renal disease. However, a relative decrease of U_{Na} is not a consistent characteristic of occlusive arterial lesions. The data emphasize the need for radiologic visualization of the main renal arteries in the diagnosis of unilateral and bilateral occlusive lesions and of combinations of arterial and parenchymal disease since these may unpredictably affect urine volume and composition.

34. VOLUME CHANGES IN THE FOREARM VEINS OF MAN DURING VOLUNTARY HYPERVENTILATION

JOHN W. ECKSTEIN, M.D., JOHN M. MCCAMMOND, B.A., AND WILLIAM K. HAMILTON, M.D., IOWA CITY, IOWA

(INTRODUCED BY JAMES W. CULBERTSON, M.D.)

Hyperventilation caused an increase in cardiac output and forearm blood flow, a fall in arterial pressure and circulation time, and no change in central venous pressure (Burnum, Hickam, McIntosh). Venous dilatation during this reported arteriolar dilatation would be inconsistent with the increased output and unchanged central pressure. Our study demonstrates and quantitates the forearm venous responses to hyperventilation.

Observations on end-expiratory pCO_2 (pCO_2), minute-volume ventilation, forearm venous distensibility (VD), and dependent forearm venous pressure (VP) were made before, during, and after voluntary hyperventilation in 16 experiments on 12 normal supine men. The venous pressure-volume curve obtained during the plethysmographic measurement of VD expresses the forearm venous volume existing at *any* effective venous pressure (EVP) between 0 and 30 mm. Hg. The naturally occurring venous volume (VV) is the volume coordinate of the point on the curve corresponding to the *measured* VP (= EVP) in the forearm unencumbered by the plethysmograph. VD is designated arbitrarily as the venous volume at an EVP of 30.

During hyperventilation, pCO_2 averaged 22.4 mm. Hg and minute-volume ventilation averaged 31.7 L./min. VD averaged 4.1 c.c./100 c.c. of forearm tissue (range, 3.1 to 5.7) during control periods and fell to 3.3 (range, 1.9 to 4.9) during hyperventilation. VP decreased in 12 and increased in 4 experiments; the average change was a fall of 1.2 mm. Hg. VV fell from an average control value of 2.9 c.c./100 c.c. of forearm tissue (range, 1.5 to 3.5) to 1.9 (range, 0.4 to 2.8)—a venous volume reduction of 35 per cent. Recovery from this uniformly observed venoconstriction was incomplete until pCO_2 returned to control levels. The VV decrease observed with room air hyperventilation was prevented partially in 4 of 5 cases by increasing inspired pCO_2 (preventing hypocapnia).

These experiments demonstrate that forearm venous dilatation does not accompany the reported forearm arteriolar dilatation observed during hyperventilation; on the contrary, the forearm veins constrict significantly during this stimulus. This degree of peripheral venoconstriction appears adequate to provide the increased venous return required for the reported increase in cardiac output and is consistent with the unchanged central venous pressure.

35. A DISCREPANCY BETWEEN TYPE OF GONAD AND THE CHROMATIN PATTERN: "OVARIES" WITH CHROMATIN-NEGATIVE (MALE) SOMATIC CELLS

W. W. Engstrom, M.D., F. J. Stoddard, M.D. (By Invitation), and E. G. Bertram, Ph.D. (By Invitation), Milwaukee, Wis.

Several different observers have reported the finding of a female chromatin pattern in most cases of apparent males with seminiferous tubule dysgenesis (so-called Klinefelter's syndrome). In some of these even gametogenesis of the male type has been observed. This finding appears to represent the first demonstrated example of a complete sex reversal in humans because gonadal sex (when it can be identified) corresponds to the sex chromatin pattern. The reverse situation, i.e., the finding of ovaries containing follicles and germinal cells in an apparent female with a male type of chromatin pattern has not yet been reported. This communication reports such a case; the patient differed from most cases of "gonadal agenesis" (Wilkins) in that the patient was not short, had no recognizable congenital anomalies, and the urinary FSH was not elevated.

The 24-year-old slender, farmer's wife had primary amenorrhea, measured 5 feet, 5½ inches, had sparse sexual hair, virtually absent breast development, and hypoplastic vaginal mucosa, low 17-ketosteroid excretion (2 to 4 mg./24 hr.), absent urinary FSH (less than 6 M.U./24 hr.), hypoplasia of feminine external genitalia and uterus, well-developed broad ligaments and Fallopian tubes, "ovaries" (albeit poor quality) containing primordial follicles, a few germinal cells, atretic follicles and an increase in fibrocollagenous tissue. Cells of skin and buccal mucosa were chromatin-negative (male).

The discrepancy between the type of gonad and chromatin pattern, as exemplified by this case and by cases of so-called Klinefelter's syndrome, presents a serious challenge to current theories of sex differentiation and the histologic method of "sexing." We tentatively suggest that if a genetically determined chemical inductor or "evocator" influences the primitive genital ridge to develop into either testis or ovary and if the nature of the gonad can be *opposite* to that of the cellular chromatin pattern, then the chromatin pattern (at least under these circumstances of abnormal sexual development) does not accurately reflect the qualitative preponderance of male or female "evocator." Chromatin-negativity in our case may not represent "maleness" but merely lack of it and female preponderance.

36. LATENT ADENOVIRUS INFECTIONS OF THE HUMAN RESPIRATORY TREE

Alfred S. Evans, M.D., Madison, Wis.
(With the Technical Assistance of B. Evans and G. Gogat)

The presence, frequency, and mechanism of latent virus infections of different parts of the human respiratory tree have been studied in order to understand better the possible role such agents might play in recurrent respiratory illnesses. To this end human tonsil, lung, and lymph node tissue removed at surgery have been cultured for prolonged periods of time up to 9 months in a roller drum, observed for cytopathogenic changes, and the supernatant fluid tested for virus at frequent intervals in HeLa and other cells. Tissue from

30 tonsils, 9 lungs, and 4 lymph nodes have been successfully cultivated. Adenovirus has been recovered from 15 tonsils and 1 lung after periods of tissue growth averaging 30 days and ranging from 8 to 70 days. No other virus has been clearly identified. Isolations were most frequent in the 6 to 10 age groups. Adenovirus types recovered included four type 1, eleven type 2, and one type 5. The serum from all but two of the individuals from whose tissue virus was recovered contained demonstrable neutralizing and complement-fixing antibodies to the type isolated. In the 2 exceptions there had been a long delay in the emergence of virus from tonsil tissue suggesting a weak antigenic stimulus. In the serum from individuals from whom no virus was isolated antibody was present in some, absent in others.

Factors bearing on the demonstration of virus and its relation to latency and "masking" were studied in detail. Adenovirus could not be recovered directly from the original material itself even after mincing or homogenization. It could not be demonstrated by implanting pieces of the original tissue directly on to HeLa cells. Virus was found under most circumstances only by growing the tissue itself for about a month and continued to be present for periods up to 70 days. Further data have been obtained indicating that the term "masking" is probably not appropriate to this phenomenon, which is largely explicable on a quantitative basis.

37. THE CLEARANCE OF HETEROLOGOUS PROTEIN FROM THE CIRCULATION OF NORMAL AND IMMUNIZED MAN

ANTHONY P. FLETCHER, M.D. (BY INVITATION), NORMA ALKJAERSIG, M.S. (BY INVITATION), AND SOL SHERRY, M.D., ST. LOUIS, MO.

The clearance rates of intravenously administered antigenic protein have been extensively studied in normal and immunized animals, but hitherto no such studies have been made in man. Previously only large doses of antigen (mg./Kg. body weight) have been used and relatively little attention has been given to the early phases of protein removal when antigen-antibody union in the circulation might be expected to influence the results. Consequently the events occurring during the early phases of immunologic insult in man have remained obscure.

Streptokinase, a product of hemolytic streptococcal metabolism, is antigenic to man and as a consequence of naturally occurring infections, the population exhibits varying levels of specific circulating antibody toward this substance. Preparations of streptokinase, homogeneous by biophysical examination, but showing traces of impurity on immunochemical analysis were trace-labeled with I^{131} without loss of biochemical activity or detectable change in physical properties.

The patient's total circulating antibody was calculated and clearance studies were made during states of both relative antibody and relative antigen excesses. Streptokinase was removed from the circulation at a rapid exponential rate (average 50 per cent plasma clearance time 18 minutes) when doses less than those required to combine with the total circulating antibody were injected intravenously. Larger doses, greater in amount than those required to neutralize circulating antibody, showed complex clearance curves. Analysis revealed these curves to consist of two exponential component rates. The "fast" exponential component was similar to that found earlier during clearance

studies conducted during states of antibody excess (vide supra) and the "slow" component had an average 50 per cent plasma clearance rate of 81 minutes. The two rates differed significantly ($p < 0.001$), the "fast" rate being found during antigen clearance in the presence of specific antibody and the "slow" rate in its absence.

The mechanisms of antigen removal revealed by this study may be of general biologic importance since the experimental conditions used (a soluble antigen-antibody system and a dosage of only a few micrograms antigen per kilogram of body weight) would appear to be analogous to events occurring in human disease states.

38. ELECTRONMICROSCOPIC STUDIES OF RENAL BIOPSIES TAKEN FROM NEPHROTIC PATIENTS BEFORE AND AFTER DIURESIS

Giuseppe Folli, M.D. (By Invitation), Victor E. Pollak, M.B., M.R.C.P.E. (By Invitation), Ross T. Reid, M.D. (By Invitation), Conrad L. Pirani, M.D. (By Invitation), and Robert M. Kark, M.D., F.R.C.P., Chicago, Ill.

Biopsies from the kidneys of 3 healthy and 8 nephrotic individuals were studied by electronmicroscopy, by oil immersion light microscopy using ultra thin sections, and also by standard histologic techniques.

Electronmicroscopically, in healthy human glomeruli, lamina densa (basement membrane), podocytes (epithelial cells), and endothelial cells were clearly defined. The lamina densa was ± 80 mμ thick, homogeneous, structureless, and separated on either side by a clear (± 5 mμ) space from endothelial cytoplasm and podocytic processes (pedicels). Each pedicel was attached separately to the lamina densa. The lamina fenestrata (endothelial cytoplasm) completely invested the inner or capillary surface of lamina densa.

In one patient, with diffuse thickening of basement membrane by light microscopy, the lamina densa was greatly widened, and split longitudinally. The podocytes and their pedicels were normal. This anatomic picture is in accord with the concept that proteinuria in the nephrotic syndrome results from increased permeability of a damaged basement membrane.

However, in most cases the endothelial cells and lamina densa were completely normal. The podocytes were quite abnormal. Their pedicels had disappeared, and in their place a continuous layer of cytoplasm invested the lamina densa. The podocytic cytoplasm was vacuolated. Minute osmiophilic fragments, apparently podocytic in origin, were observed lying free in the capsular or Bowman's space. Three cases were studied before and after diuresis. For example, in one case proteinuria of ± 15 Gm. per day decreased to ± 2 Gm. per day after diuresis induced by prednisone. In this instance a second biopsy taken after diuresis revealed that the podocytes were almost normal; normal discrete pedicels were now seen. These observations indicate that the podocyte may play a role relative to the ultrafiltration of plasma, previously attributed by some to the endothelial cells and basement membrane.

It has been suggested that the nephrotic syndrome is the result of immune reactions in the glomerulus—localized in the basement membrane. Our findings do not support a predominant role of glomerular basement membrane changes in the pathogenesis of these cases of nephrotic syndrome, but rather emphasize the importance of glomerular epithelial cell alterations.

39. MECHANISMS OF ACTION OF DIURETICS AS REVEALED BY POTENTIATION STUDIES

RALPH V. FORD, M.D., HOUSTON, TEXAS

(INTRODUCED BY JOHN H. MOYER, M.D.)

Initial experiments in laboratory animals by Pitts (personal communication) suggested that the mechanisms of three groups of diuretics were noncompetitive. Utilizing patients with previous congestive heart failure, we have observed the acute excretory responses of sodium, potassium, chloride, and bicarbonate following the parenteral administration (priming and sustaining doses) of meralluride (Mercuhydrin), acetazoleamide (Diamox), and chlorothiazide (Diuril), separately and sequentially in various combinations.

After a maximum increase in sodium excretion following meralluride injection was observed, the administration of acetazoleamide produced another significant increase (twofold) indicating that two noncompetitive mechanisms for the tubular reabsorption of sodium had been inhibited. At the peak of this second increase, and while meralluride and acetazoleamide were still being infused, the administration of chlorothiazide was observed to produce a third significant increase (twofold) over the previous rate of sodium excretion, suggesting that a third mechanism had been affected.

To further elaborate on the competitive nature of these three mechanisms of diuretic action, the order of administration of the drugs was changed. If acetazoleamide followed the administration of chlorothiazide, no augmentation of sodium excretion was observed. Since at least part of the pharmacodynamics of chlorothiazide involves carbonic anhydrase inhibition, one may conclude that it is capable of acting on this enzyme in a competitive fashion with reference to acetazoleamide. But since chlorothiazide has other pharmacodynamic properties its effect on sodium excretion cannot be blocked by acetazoleamide. If meralluride administration in equipotent doses followed the administration of chlorothiazide, no augmentation of sodium excretion was observed, but the converse is not true. Therefore, chlorothiazide appears to be capable of competing with the mechanism for sodium excretion that is affected by meralluride.

Thus, the data indicate that meralluride shares a mechanism of action with chlorothiazide, that acetazoleamide shares a mechanism with chlorothiazide, and that chlorothiazide can act through a third noncompetitive mechanism.

40. OBSERVATIONS ON THE PATHWAYS OF CHOLESTEROL EXCRETION IN MAN

IVAN D. FRANTZ, JR., M.D., AND JAMES B. CAREY, JR., M.D., MINNEAPOLIS, MINN.

(INTRODUCED BY F. W. HOFFBAUER, M.D.)

The classic concept of the mechanism by which cholesterol is removed from the body was that it is excreted through the intestinal mucosa, converted to coprastanol in the lumen, and eliminated in the feces. More recent experiments with isotopes have been interpreted as favoring the conversion to bile acids as the major pathway of excretion. We have been reluctant to accept this notion because we have been unable to isolate from feces the quantities of bile

acids and their derivatives predicted from the rate of cholesterol turnover, and because feces from subjects fed sterol-free diets contain sizable amounts of digitonin precipitable material, the source of which is obscure if it does not represent cholesterol excreted from the blood. For these reasons, we undertook the present experiments.

A healthy man was fed a formula diet of skim milk, sugar, and chocolate. Twenty microcuries of cholesterol-4-C^{14} were given intravenously. Stools were collected daily for isolation and quantitation of the various excretion products of cholesterol, and for comparison of their specific activities with that of the blood cholesterol.

The stools were homogenized and extracted exhaustively with boiling ethanol. This treatment removed more than 99 per cent of the radioactivity. About 70 per cent of the radioactivity was extractable into petroleum ether after hydrolysis with alcoholic KOH. Of this, 80 per cent was precipitable with digitonin. About 600 mg. of digitonin precipitable sterols could be isolated daily, and these had a specific activity equal to that of the blood cholesterol two days earlier. Only 15 per cent of the daily excretion of radioactivity was recovered in the bile acid fraction, two thirds of which was isolated as deoxycholic acid.

We feel that these results, together with other evidence (e.g., J. Clin. Invest. **36**: 898, 1957), demand that further consideration be given to the possibility that the older views on the mechanism of cholesterol excretion were correct.

41. A COMPARISON OF THE USEFULNESS OF I^{131} TRIOLEIN, RISA, AND FAT AND NITROGEN BALANCE TECHNIQUES IN THE DIAGNOSIS OF MALABSORPTION

A. B. French, M.D. (By Invitation), M. Ishikawa, M.D. (By Invitation), H. S. Wiggins, Ph.D. (By Invitation), and H. M. Pollard, M.D., Ann Arbor, Mich.

Triolein and human albumin (RISA), tagged with I^{131}, are now widely used as tools for the diagnosis of malabsorption syndromes. This is based on the demonstration by several laboratories that differences in absorption of these substances can be used to separate normal from grossly abnormal patients. The efficiency and reliability of these methods as compared with traditional fat and nitrogen balance studies have not yet been clearly demonstrated.

The present study compares the results of 5-day fat and nitrogen balances with the results of fecal, urinary, and blood radioactivity determinations following administration of I^{131} triolein or RISA, and with simultaneous vitamin A and glucose tolerance curves in over 50 subjects. Diseases studied include nontropical sprue, Whipple's disease, pancreatic insufficiency, postgastrectomy steatorrhea, regional enteritis, and ulcerative colitis. In general, the correlation between increased fecal radioactivity and excessive fecal fat and nitrogen was good. Sensitivity in demonstrating abnormality was greater with triolein than with RISA. A small but important group of patients showed striking lack of correlation between radioactivity and chemical determinations. Lack of correlation was greater and more frequent when blood or

urinary radioactivity was compared. It is concluded that fecal radioactivity determinations are more useful than blood or urine determinations and that none of these show complete agreement with chemical balance studies.

42. GONADAL AND ADRENAL STEROID-INDUCED CHANGES IN THE CHOLESTEROL AND PHOSPHOLIPID CONTENT OF SERUM LIPOPROTEIN FRACTIONS ISOLATED BY SIMULTANEOUS CENTRIFUGATION AT FOUR SOLVENT DENSITIES FOLLOWED BY QUICK-FREEZING AND TUBE-SAWING

ROBERT H. FURMAN, M.D., R. PALMER HOWARD, M.D., AND LEONARD N. NORCIA, PH.D. (BY INVITATION), OKLAHOMA CITY, OKLA.

A simple technique for separation of serum lipids according to the specific gravity of the lipoprotein complex is described. Serum aliquots are spun at 40,000 r.p.m. (144,700 × g) for 22 hours at the following densities: 1.006, 1.019, 1.063, and 1.21 g per ml. The tubes are removed from the rotor without agitation and "quick-frozen" in a solution of dry ice and acetone. The plastic tube and its solid contents are sawed through in the clear zone beneath the supernatant fraction. The infranatant and supernatant fractions are then placed in funnels in volumetric flasks, allowed to melt and made up to standard volume with methanol-methylal. Lipid analyses are then carried out on the filtrates.

Five subjects were given peroral methyltestosterone, one equilin sulfate, three prednisone, and one cortisone acetate.

The cholesterol and phospholipid content of native serum and of each density-fraction was determined, and the percentage distribution of these lipids in each fraction calculated.

Equilin sulfate, cortisone, and prednisone produced absolute and relative increases in the amount of lipid in the highest density $> 1.063 < 1.21$ g per ml. ("alpha") fraction, while methyltestosterone reduced the lipid content of this fraction.

The lipid content of the $> 1.019 < 1.063$ ("beta") lipoprotein fraction showed a less consistent response. Equilin sulfate and cortisone reduced the cholesterol and phospholipid content of the beta lipoprotein fraction, both relatively and absolutely. Methyltestosterone administration led to increases in beta lipoprotein cholesterol and phospholipid with one exception when no change was noted.

Increased beta lipoprotein lipid following prednisone administration was noted only when marked increase in the native serum cholesterol and lipid phosphorus occurred. When no increase in native serum lipid concentration was noted following prednisone administration, beta lipoprotein lipid concentration fell, absolutely and percentagewise.

The cholesterol and phospholipid content of the > 1.006, < 1.019, and < 1.006 fractions either fell or underwent no change during equilin sulfate or adrenocorticosteroid administration, whether or not native serum lipid concentrations increased; the response following methyltestosterone was slight and inconsistent.

The effects of adrenocorticosteroids on serum lipids and lipoproteins do not appear to be a consequence of thyroid suppression.

43. STUDIES IN THE PATHOGENESIS OF LUPUS ERYTHEMATOSUS: EXPERIMENTAL PRODUCTION OF HEMATOXYPHIL BODIES IN THE KIDNEY

JAMES L. GERMAN, M.D., BETHESDA, MD.

(INTRODUCED BY F. J. HECK, M.D.)

Observations under phase contrast microscopy of unstained preparations containing L.E. cells and L.E. bodies disclosed that these structures, in addition to being relatively rigid and "sticky," were often larger than normal formed elements of the circulating blood. This suggested that obstruction to small blood vessels could develop if such structures should circulate in the blood stream.

These observations, combined with the known histochemical identification of in vitro L.E. bodies with lupus tissue "hematoxyphil" bodies and the unique pathognomonic significance of the latter, prompted animal experimentation.

Suspensions of L.E. cells and L.E. bodies were injected into the aortas of rabbits and guinea pigs. The kidneys, removed and fixed from 15 minutes to 4 hours after injection, showed an abundance of structures in the glomerular capillaries which by contour, texture, and staining reactions (purple on H and E stain; Feulgen positive) seemed characteristic of the hematoxyphil, or "hematoxylin," bodies found in the lupus kidney. Control animals were negative.

44. THE EFFECT OF BLOCKING DRUGS ON THE VASCULAR REACTION TO ENDOTOXIN IN CATS

ROBERT P. GILBERT, M.D., MINNEAPOLIS, MINN.

Histamine, 5-hydroxytryptamine (5-HT) and epinephrine have been suggested as mediators of the vascular responses to bacterial endotoxin. Chlorpromazine and dibenzyline, which block the effects of one or more of these drugs, have been shown to protect mice against the lethal effects of endotoxin. The present study concerns the effect of similar blocking drugs on the early vascular responses to endotoxin.

Carotid (CAP) and pulmonary (PAP) arterial pressures were measured in the open chested cat under pentobarbital anesthesia. The administration of endotoxin to the cat characteristically causes a PAP rise and a CAP fall. Because antihistaminics and dibenamine did not block the vascular effects of large doses (0.1 to 1.0 mg./Kg.) of histamine and 5-HT, a dose of endotoxin was chosen which did not produce an overwhelming effect.

In 15 control cats, 0.05 mg./Kg. of endotoxin caused a CAP fall of 45 per cent (± 9 s.e.) and a PAP rise of 73 per cent (± 17 s.e.). The combination of dibenamine (15 mg./Kg.) and pyrilamine (5 mg./Kg.) afforded almost complete protection in 5 cats: the CAP *rose* 3 per cent (± 7 s.e.) and the PAP rose 27 per cent (± 10 s.e.). In 5 trials of each agent alone there was only partial protection. The combination of LSD (0.5 mg./Kg.), with no adrenolytic activity, and pyrilamine gave fair protection in 4 cats: the average CAP fall was 12 per cent and the average PAP rise was 15 per cent. LSD alone mitigated the fall in systemic pressure, but did not alter the PAP rise. The effect of large doses of endotoxin (5 mg./Kg.) was not appreciably lessened by these drugs.

These results are consistent with the possibility that histamine and 5-HT are active during the early phase of the endotoxin reaction. It does not

follow that the blocking agents used would be of therapeutic use, especially during the late phases of a severe endotoxin reaction.

45. SOME OBSERVATIONS ON THE VARIABLE FATE OF BILIRUBIN DEPENDING ON CONJUGATION AND OTHER FACTORS

SIGRID GILBERTSEN, M.D. (BY INVITATION), MALCOLM CAMPBELL, M.D. (BY INVITATION), AND C. J. WATSON, M.D., MINNEAPOLIS, MINN.

Earlier studies had shown that crystalline bilirubin given per duodenal tube in patients with complete biliary obstruction was largely unaccounted for as urobilinogen in the feces. In the present study intraduodenal administration of N^{15} crystalline bilirubin in 3 cases of carcinomatous biliary obstruction and in a normal subject was followed by recovery of less than 5 per cent as fecal urobilinogen and no increase of mesobilifuscin (dipyrrylmethene group). Crystalline bilirubin given intravenously in a normal subject was largely accounted for (79 per cent) as urobilinogen, with minor amounts of mesobilifuscin and bilirubin. The administration of a crude bilirubin glucuronide per duodenal tube in a normal subject was entirely accounted for as fecal urobilinogen and mesobilifuscin, in the normal ratio. Substantial increases in fecal urobilinogen were noted in similar experiments in two patients with complete biliary obstruction.

A remarkable contrast to the normal handling of bilirubin was observed in a patient with refractory anemia. The Cr^{51} red cell survival measurement was normal, but the fecal urobilinogen was regularly very small, averaging but 22 per cent of the anticipated value. Here there was no significant increase of fecal bilirubin, urobilinogen, or dipyrrylmethene following administration of bilirubin glucuronide intraduodenally or crystalline bilirubin intravenously. Yet when crystalline mesobilirubinogen (*i*-urobilinogen) was given intraduodenally, the increase of fecal urobilinogen corresponded with the amount administered. These observations suggest that in this patient there is an aberrant fate of bilirubin, but whether in the intestinal tract is not clear. That it is not due to failure of conjugation of bilirubin by the liver was evidenced by the usual prompt direct diazo reaction of the bilirubin in the duodenal contents.

The present studies thus indicate that while conjugation of bilirubin is important or even essential to its normal conversion to urobilinogen by the fecal flora, there may exist significant deviations of the ordinary pathway based on other mechanisms which are not yet clear. Whether these are fundamentally related to disease states, such as anemia, also remains to be determined.

46. BLOOD CHOLESTEROL LOWERING EFFECT OF ESSENTIAL FATTY ACID SUPPLEMENTS IN DIABETIC PATIENTS

EDGAR S. GORDON, M.D., MADISON, WIS.

Preoccupation with atherosclerosis as an important human disease accounts for the widespread interest in cholesterol and a variety of methods that have been devised to alter blood cholesterol levels. Of these methods, one of the most interesting has been the addition of essential fatty acid supplements (EFA) to the diet. Special interest in diabetic subjects has resulted from (1) the well-recognized accelerated rate of development of atherosclerosis in diabetics, (2) the report of Holman that diabetic animals have an EFA requirement significantly higher than that of normal controls, and (3) the tendency for many diabetics to have elevated blood cholesterol levels.

Fourteen severe diabetic patients of both sexes, ranging in age from 19 to 54, have been studied over a period of 7 months. All subjects are suffering from diabetic vascular complications of the Kimmelstiel-Wilson type and many have severe loss of vision. EFA has been administered in the form of safflower oil in a dose of 30 ml. morning and evening, accompanied by an isocaloric reduction in the fat of their diabetic diets. Measurements have been made serially of total, free and esterified cholesterol, total content, and iodine number of circulating plasma fat. Careful clinical observations have accompanied these laboratory studies.

Blood cholesterol levels have been consistently and significantly lowered in all subjects. No significant change in the free/ester ratio has been noted. Total circulating blood lipids have risen progressively in most instances and no consistent change (rise) in iodine number of these fats has occurred. All patients have been improved clinically. No spontaneous retinal hemorrhages have occurred in any patient since EFA supplements were started and tourniquet tests, initially strongly positive, have become less positive and in a few instances, negative. Insulin requirements have tended to decrease slightly. The possible significance of these observations will be discussed.

47. RETROGRADE AORTIC CATHETERIZATION IN AORTIC STENOSIS

E. W. Green, M.D. (By Invitation), and R. F. Ziegler, M.D., Detroit, Mich.

The precise determination of various degrees of aortic stenosis requires direct measurement of the pressure gradient across the valve. It seemed technically advantageous, particularly in infants and children, to measure this gradient by retrograde catheterization of the aorta and left ventricle from the right brachial artery. This procedure was performed successfully, without morbidity and mortality in 15 cases of aortic stenosis ranging in age from 10 weeks to 22 years. From this approach it was possible to explore both ascending and descending aorta, which proved the pressure, location, and degree of associated coaretation in 3 cases. In entering the left ventricle constant care with fluoroscopic visualization and simultaneous pressure monitoring was exerted to first outline the exact position of the valve and then to pass it without traumatizing the coronary arteries. Ventricular irritability was notably absent as long as catheterization of this chamber was done with care.

In addition to the quantitative physiologic information provided, this technique has established by electrocardiographic correlation a foundation for the more accurate clinical determination of the presence, degree, and hemodynamic type of left ventricular enlargement. In combination with right heart catheterization it has also proved to be of importance in detecting and evaluating the presence of combined valve lesions such as pulmonary and aortic or mitral and aortic stenosis, particularly when other signs such as those indicative of combined cardiac chamber enlargement may be equivocal or absent.

48. A PITUITARY-PROSTATE RELATIONSHIP NOT MEDIATED BY THE TESTIS AS REVEALED BY STUDIES WITH TESTOSTERONE-4-C^{14} IN THE MALE RAT

David S. Greer, M.D., Chicago, Ill.

(Introduced by Richard L. Landau, M.D.)

After the subcutaneous administration of a physiologic dose of testosterone-4-C^{14} to intact, castrated, and hypophysectomized castrated, pubescent male rats,

greater concentrations of radioactivity were detected in the prostate and other male accessory glands than in nonsex-linked tissues. The evidence gathered suggests that the radioactivity detected in the prostate represented the androgen or a rather immediate metabolic thereof.

In intact rats and in rats castrated 2 or 3 days the prostatic concentration of radioactivity averaged 2.5 to 3.0 times that in tissues regarded as "androgen independent" (e.g., tongue). The radioactive isotope could not be removed by perfusion with normal saline. Cannulation of the bile ducts to eliminate hepatic metabolites and intestinal degradation products of testosterone did not diminish the avidity of prostatic tissue for the isotope. The injection of cholesterol-4-C^{14} as a control steroid did not yield similar concentrations of tissue radioactivity.

Hypophysectomy 3 days prior to the administration of testosterone-4-C^{14} lessened the attraction of the prostate for the androgen derived isotope. The mean concentration of radioactivity in the prostates of 2-day castrated animals was 14.7 disintegrations per minute per 10 mg. wet weight, whereas the mean concentration in prostates of hypophysectomized castrated animals was 9.9 disintegrations per minute per 10 mg. wet weight. A comparison of the ventral prostate/tongue ratios of radioactivity (a device employed to cancel the effects of variations in the concentration of circulating isotope on its localization in both prostate and "androgen-independent" tissues, as well as to emphasize the "specific" localization in prostate) delineated the two groups of animals even more sharply. The mean ventral prostate/tongue ratio in castrates was 2.87; in hypophysectomized castrated rats, 1.62 (p value < 0.001).

These data suggest that the localization of androgen by male accessory tissues may be an important facet in the endocrine control of their growth and metabolism. The impairment of this localizing process in hypophysectomized castrated rats points to a mechanism by which the pituitary could influence the prostate by means other than stimulation of the testis.

49. INHIBITORY EFFECTS OF STEROIDS ON THE GROWTH OF UTERINE FIBROBLASTS: THE ISOLATION OF STEROID-RESISTANT VARIANTS

B. I. Grosser, and H. E. Swim, Ph.D., Cleveland, Ohio

(Introduced by R. F. Parker, M.D.)

The effect of steroids on the proliferation of mammalian cells cultured in vitro has been investigated. Uterine fibroblasts, strain U12-79, were propagated in a defined medium supplemented with 5 per cent dialyzed horse serum (medium 79). To examine the effects of steroids on growth, U12-79 was propagated serially for periods of 3 weeks (3 subcultures) in medium 79 containing a single steroid in concentrations ranging from 0.1 to 25 μg per milliliter. Cells exposed to corticosterone, desoxycorticosterone, estradiol, estriol, hydrocortisone, progesterone, or testosterone at a concentration of 25 μg degenerated over a period of 2 to 7 days. Hydrocortisone and corticosterone inhibited growth at concentrations as low as 0.1 μg per milliliter whereas the remaining steroids reduced proliferation only at levels of 5 μg per milliliter. Inhibition of growth in the presence of steroids was accompanied by cytologic changes. In view of the fact that a population of U12 cells has been shown to contain nutritional variants (Swim, H. E., and Parker, R. F.: Fed. Proc. **16:**

435, 1957) the possibility that steroid resistant variants were also present was investigated. Cells were treated with either hydrocortisone or testosterone at a concentration of 25 μg per milliliter. Under these conditions, the cells gradually degenerated until it appeared that all of the cells were involved. The regular schedule of fluid replacements was continued and subsequently a few healthy cells were noted in the cultures and their progeny continue to proliferate at essentially the same rate in the presence of steroid as that of the U12-79 strain in medium 79. Previous experiments (Sweat, M. L., Grosser, B. I., Nabors, C. J., and Swim, H. E.: Fed. Proc. **16**: 258, 1957) indicate that hydrocortisone is metabolized by strain U12-79. Studies are now under way to determine whether the metabolism of hydrocortisone by the resistant variant differs from that of the sensitive U12-79 strain.

50. THE STIMULATORY EFFECT OF "ANEMIC PLASMA" IN CONGENITAL HYPOPLASTIC ANEMIA

Clifford W. Gurney, M.D. (By Invitation), Mila I. Pierce, M.D. (By Invitation), Stanley E. Schrier, M.D. (By Invitation), Paul E. Carson, M.D. (By Invitation), and Leon O. Jacobson, M.D., Chicago, Ill.

Congenital hypoplastic anemia is a chronic anemia appearing in the first few months of life, characterized by a normochromic anemia, persistently low reticulocyte count, and marrow hypoplasia confined to the erythrocytic series. It is usually refractory to therapy other than repeated transfusions. Our studies of the hormonal regulation of erythropoiesis led us to consider the possibility that a deficiency of an essential plasma factor might be responsible for erythroid hypoplasia in this disease. This hypothesis was tested by observing the effects of "anemic plasma" in two patients with congenital hypoplastic anemia. "Anemic" rather than normal plasma was used because we demonstrated previously the increased erythropoietic-stimulating properties of such plasma. Donors of the same blood type as the patients were bled until their hematocrit values reached 25 per cent. "Anemic plasma" was then harvested daily, the packed cells being returned to the donors to prevent further fall in their hematocrits.

Daily infusions of approximately 300 ml. of "anemic plasma" were given to two patients with congenital hypoplastic anemia. After 5 infusions, the reticulocyte count exceeded any values recorded previously for either patient, rising to 4.0 per cent after 8 infusions in one patient and 3.4 per cent after 6 infusions in the other. Iliac marrow aspirations were performed prior to the onset and again upon termination of the course of the infusions. In the first patient, who responded with a transient rise in hemoglobin as well as a reticulocytosis, the marrow showed increased numbers of proerythroblasts and all stages of normoblasts. In the second patient, no rise in hemoglobin accompanied the reticulocytosis, and marrow changes were less striking although early normoblasts were increased.

We conclude that some factor present in "anemic plasma" is capable of stimulating erythropoiesis in congenital hypoplastic anemia. This finding suggests congenital hypoplastic anemia is the result of a congenitally-determined deficiency of some essential plasma factor. Current investigation is directed toward elucidating the nature of this factor and its possible relationship to erythropoietin, the erythrocyte-stimulating hormone.

51. EFFECT OF CHANGE IN BLOOD FLOW RATE UPON RENAL VASCULAR RESISTANCE

F. Haddy, M.D., Ph.D., J. Scott, M.S., M. Fleishman, M.D., and D. Emanuel, M.D., Fort Knox, Ky.

(Introduced by Craig W. Borden, M.D.)

It has long been known that renal vascular resistance increases progressively as arterial pressure is raised in the range 80 to 200 mm. Hg. This accounts for the unique capability of the kidney to keep its blood flow rate constant despite large changes in arterial pressure. The response has been variously ascribed to active vasoconstriction, passive vasoconstriction, absolute blood viscosity increase, and dynamic blood viscosity increase. In an attempt to define the mechanism responsible for the resistance increase, the effect of changing flow rate in the range 25 to 180 ml./min. upon renal vascular resistance was studied in 82 pentobarbitalized laparotomized dogs. Flow rate was varied by interposing a blood pump in the right renal artery. On the average, resistance progressively decreased when blood flow rate was raised from 25 to 75 ml./min. and progressively increased when raised from 75 to 180 ml./min. However, intraindividual and interindividual comparisons showed the onset of the resistance increase to be flow rate independent but pressure dependent. The onset always occurred at mean arterial pressures between 50 and 100 mm. Hg regardless of the flow rate. This relationship between resistance and pressure was unchanged by extrinsic nerve section, destruction of circulating catecholamines, local sympathetic nerve blockade, local parasympathetic nerve potentiation, increased CO_2 tension, and $[H^+]$ and decapsulation. The resistance increase was abolished and replaced by a progressive resistance decrease during blood perfusion when dextran was intermittently used as the perfusate. The resistance increase was not observed in dextran perfused kidneys and in blood perfused dead kidneys. Elevation of arterial pressure by increasing blood flow rate failed to significantly increase lymph flow rate from cannulated hilar lymph vessels. Winton, Gottschalk, Swann, and Miles have separately shown that elevation of renal artery pressure is accompanied by no or relatively little change in interstitial pressure. These observations indicate that the resistance increase results from active vasoconstriction. The vasoconstriction appears to be caused by some effect of an increase in arterial transmural pressure acting directly upon vascular smooth muscle.

52. THE EFFECT OF ANTIBIOTIC COMBINATIONS UPON STAPHYLOCOCCI, WITH SPECIAL REFERENCE TO OLEANDOMYCIN

Wendell H. Hall, M.D., Ph.D., and Harold Elliott, B.A. (By Invitation), Minneapolis, Minn.

In a previous report we demonstrated the activity of 12 antibiotics in 66 paired combinations against 30 hospital strains of *Staphylococcus aureus*. Activity was measured by broth dilution with subcultures and by paper strip-agar diffusion with replica plating. Addition of antibacterial activities of antibiotics was frequent but true synergism and antagonism were rare with these rather resistant bacterial strains.

Recent claims for synergism of action with delay in emergence of resistance prompted studies with the new antibiotic, oleandomycin, in combination with various other antibiotics.

Though rather resistant to penicillin and tetracycline, most of the 30 strains of staphylococci were inhibited in broth by a smaller concentration of Sigmamycin (oleandomycin ⅓ plus tetracycline ⅔), AP (oleandomycin ⅓ plus penicillin G ⅔) and APG (AP ⅔ plus penicillin G ⅓) than by oleandomycin, tetracycline, or penicillin. These antibiotic combinations also showed addition of growth inhibition on agar plates when tested individually and with bacitracin, chloramphenical, erythromycin, novobiocin, neomycin, and vancomycin. Antagonism was exceedingly rare and true synergism was never observed on agar plates. Oleandomycin was less active than erythromycin.

Contrary to early reports, oleandomycin combinations proved to show little addition of antibacterial activity against resistant bacterial strains.

A few strains of susceptible staphylococci were made resistant to individual and paired antibiotics by repeated exposure to doubling concentrations in broth. Resistance developed rapidly and to high concentrations with the single antibiotics, streptomycin, erythromycin, oleandomycin, and vancomycin. Resistance to penicillin and to neomycin developed slowly and to relatively low concentrations with most bacterial strains, though a few rapidly became highly resistant. Mixtures of equal parts of these antibiotics in pairs were also used. Vancomycin with oleandomycin or erythromycin did not retard resistance. Streptomycin slowed moderately the rate of resistance to both oleandomycin and erythromycin. Contrary to expectation, oleandomycin markedly slowed the rate of resistance to the closely related antibiotic, erythromycin.

Antibiotic combinations are useful in staphylococcal infections, especially if antibiotic susceptibility tests are lacking. Addition of antibacterial activities may be helpful and emergence of resistant strains may be retarded by a proper selection of antibiotics. Mixtures of similar antibiotics may prove more useful than has been apparent hitherto.

53. CIRCULATORY DYNAMICS IN ACUTE GLOMERULONEPHRITIS

HARPER K. HELLEMS, M.D., VALENTINO DEFAZIO, M.D. (BY INVITATION), YOSHIKAZU MORITA, M.D. (BY INVITATION), RAYMOND C. CHRISTENSEN, M.D. (BY INVITATION), AND LESEM J. BAER, M.D. (BY INVITATION), DETROIT, MICH.

Although heart failure is the most common cause of death in the initial stage of acute glomerulonephritis, its pathophysiology is poorly defined. To determine the circulatory abnormalities present in this disease, hemodynamic studies, employing the technique of cardiac catheterization, have been carried out in 6 patients with typical clinical and laboratory findings of acute glomerulonephritis of a few days' duration. Patients were studied in the resting state.

At the time of study, all patients had elevated blood pressures; 5 were edematous; 2 had pulmonary congestion. In 5 of the subjects, including both patients with pulmonary congestion, the arm-to-tongue circulation time was within normal limits.

In 2 patients oxygen consumption was slightly elevated, but was normal in the remaining 4. The arteriovenous oxygen difference ranged from 25.7 to 42.5 ml./L. with a mean of 31.7 ± 2.6 (s. e. m.). The cardiac index ranged from 4.43 to 7.08 L./min./M.^{2} with a mean of 5.18 ± 0.40, a value significantly above the normal.

In all patients the pulmonary "capillary" pressure was elevated, ranging from 14 to 39 mm. Hg with a mean of 23.3 ± 3.7, indicating a high left ventricular filling pressure. The pulmonary artery and right ventricular systolic pressures were abnormally high in each and right ventricular filling pressure was significantly increased in 4 of the 6 patients.

Although all patients were hypertensive with brachial artery mean pressures ranging from 136 to 182 mm. Hg, the calculated total peripheral resistance was within normal limits (range 1,100 to 1,502 dynes/sec./cm.$^{-5}$, mean 1,303 ± 126), indicating that the hypertension in this high output state is independent of any significant reduction in the total cross-sectional area of the peripheral vascular bed. Total left ventricular and left ventricular stroke work were markedly elevated. Two patients studied after diuresis had occurred showed entirely normal circulatory dynamics.

In conclusion, in the edematous stage of acute glomerulonephritis there is a high cardiac output with associated increase in ventricular filling pressures suggesting that these patients have a high output type of heart failure.

54. CLINICAL AND METABOLIC CHANGES INDUCED BY GLUCAGON IN PATIENTS WITH RHEUMATOID ARTHRITIS

O. M. Helmer, Ph.D., W. R. Kirtley, M.D., and A. S. Ridolfo, Ph.D., M.D. (By Invitation), Indianapolis, Ind.

In our laboratories we have been studying the effects of glucagon on carbohydrate and protein metabolism. One of the most striking effects of its administration is the lowering of amino nitrogen in the blood of human subjects and experimental animals. The change in amino nitrogen of the blood is greater than that of glucose. The effect of glucagon on protein metabolism does not appear to be mediated through the adrenal glands, since in adrenalectomized rats the fall in amino nitrogen is of the same order as in the intact animal but is not as sustained.

Salter and Best have also demonstrated that glucagon has a protein catabolic effect, and on this basis they administered glucagon to patients with rheumatoid arthritis. These investigators observed that an anti-inflammatory effect accompanied the protein catabolic action.

With hope of throwing more light on the metabolic mechanisms concerned, 12.5 mg. of crystalline glucagon was given intravenously over a period of 10 hours to 3 patients with rheumatoid arthritis. Each patient received infusions for 3 consecutive days. Of the blood constituents examined, the decrease in amino nitrogen was of greatest magnitude, greater than that of rise in glucose. In the urine there was an increased excretion of nitrogen, phosphate, 17-OH corticoids, and uric acid during the infusion period with variable appearance of ketone bodies. There was minimal glycosuria.

Subjectively, all patients volunteered that there was diminished pain, increased joint motion, and a continued feeling of well-being for periods up to 7 days after the discontinuance of therapy. One patient whose disease was in the acute phase also demonstrated decreased redness of joints. Untoward symptoms were limited to nausea and loss of appetite which occurred throughout the period of infusion.

Because of the nausea and short duration of action, glucagon in its present form appears to have no practical value in the treatment of arthritis. However, it is possible that further experimental study may not only shed light on the basic mechanism of inflammation but also increase our knowledge of protein, carbohydrate, and energy metabolism.

55. PROTEIN REQUIREMENTS IN CHRONIC RENAL INSUFFICIENT PATIENTS

A Study of the Nitrogen Minimum

Richard F. Herndon, M.D. (By Invitation), Smith Freeman, M.D., Ph.D., and Anne S. Cleveland, Ph.D. (By Invitation), Chicago, Ill.

The protein requirement for nitrogen equilibrium was estimated from nitrogen balance studies on five chronic renal insufficient patients and one healthy subject. The minimum nitrogen requirement for nitrogen balance in the renal insufficient patients exceeded that of a healthy subject.

The increase in protein requirement for nitrogen balance seemed to be greatest in patients with the most advanced renal insufficiency. Whether this was due to a generally reduced efficiency in protein utilization or to increased requirements for some particular amino acids is not known.

The addition of glycine and a mixture of both D- and L-forms of the 8 essential amino acids according to Rose resulted in nitrogen loss out of proportion to the increased nitrogen intake; supplementation with L-methionine resulted in a nitrogen loss increased in proportion to the increase in nitrogen intake and no protein sparing effect could be demonstrated. The addition of a mixture of L-amino acids composed of 75 per cent leucine, 15 per cent isoleucine, 8 per cent methionine, and 3 per cent phenylalanine resulted in a modest nitrogen sparing effect in one patient and no effect in two others.

56. THE INTERPRETATION OF ELECTROLYTE CONCENTRATIONS OBTAINED DURING INTRAVENOUS ELECTROLYTE INFUSIONS

Carl R. Hines, M.D., Chicago, Ill.

(Introduced by Sheldon S. Waldstein, M.D.)

In the management of patients with electrolyte abnormalities, an intravenous repair solution is often started before blood samples are obtained for analysis. The significance of the results of these subsequent electrolyte determinations is therefore questioned. This experiment was designed to study this situation in normal subjects preliminary to study of patients with electrolyte abnormalities.

Five hundred milliliters of a solution containing 155 mEq./L. of sodium chloride and 40 mEq./L. of potassium chloride were administered intravenously to each of 20 normal, fasting adults. This was given at a constant rate in exactly 2 hours. Blood was obtained from the opposite extremity prior to and 30, 60, and 120 minutes after the start of the infusion. The microhematocrit and serum sodium, potassium, and chloride concentrations were determined in each blood specimen.

The data were submitted to an analysis of variance. The following changes were found to be statistically significant at the 5 per cent level: a decrease in the hematocrit and an increase in the serum potassium which persisted during the entire infusion; and an increase in the serum chloride concentration 1 and 2 hours following the start of the infusion. There was no significant change in the serum sodium concentration.

The maximal variations of the means were: hematocrit, 3 per cent; serum potassium, 0.38 mEq./L.; and serum chloride, 1.9 mEq./L. If true in abnormal

patients, as well, variations of this degree would be unlikely to alter the diagnostic appraisal. This demonstrates very rapid and nearly complete mixing of infused electrolytes in normal patients.

It is concluded that intravenous administration of sodium and potassium chloride at the concentrations and rate described to normal adults does not alter significantly the hematocrit or serum sodium, potassium, and chloride concentrations in blood obtained during the course of the infusion.

57. A CHROMATOGRAPHIC STUDY OF THE CHEMICAL STRUCTURE OF HUMAN SERUM LIPIDS

JULES HIRSCH, M.D., WILLIAM INSULL, JR., M.D., AND EDWARD H. AHRENS, JR., M.D., NEW YORK, N. Y.

(INTRODUCED BY IRVINE H. PAGE, M.D.)

Adsorption chromatography with silicic acid is a valuable method for separating the components of many complex lipid mixtures. Recent modifications of this method involving the special selection and preparation of adsorbent and the use of gradient elution render it particularly applicable to the separation and chemical characterization of the components of human serum lipids. Preliminary studies with other adsorbents indicate that such chromatographic separations may be accomplished by a rapid and automatic means.

These techniques demonstrate the existence of the following classes of lipids in the serum: hydrocarbons (carotene), cholesterol esters, triglycerides, nonesterified fatty acids, cholesterol, and phospholipids. The phospholipids are further separable into three substances corresponding to: cephalins, lecithin, and sphingomyelin. In a large number of normal and abnormal sera, no diglycerides or monoglycerides can be found; but, during the heparin-induced clearing of lipemic serum, small amounts of these substances are present.

The complete separation and quantitative recovery of cholesterol esters, triglycerides, and phospholipids enable the isolation of the fatty acids present in each of these groups. The further analysis of these fatty acids by gas-liquid chromatography completes the chemical definition of the serum lipids.

Previous studies have shown that the formula feeding of single, well-defined fats to human subjects produces predictable alterations in the concentration of the serum lipid classes. The complete analysis described above shows striking variation of the fatty acid composition of each lipid class in response to such dietary manipulations. This is most marked in the triglyceride fatty acids, but the cholesterol esters and phospholipids also show distinct changes. Thus, the feeding of a corn oil formula following a butter formula more than doubled the linoleic acid content of the cholesterol esters and phospholipids, but the amount of linoleic acid in the triglycerides was increased *eleven* fold.

58. APPARENT STIMULATION OF THYROID ACTIVITY IN MEN FED AN UNUSUAL DIET

ROBERT E. HODGES, M.D., AND TITUS C. EVANS, PH.D., IOWA CITY, IOWA

(INTRODUCED BY HENRY E. HAMILTON, M.D.)

A group of 6 healthy young men was fed a formula composed of purified casein, corn oil, carbohydrates, vitamins, and minerals. This strange diet has been used by us in previous studies of pantothenic acid deficiency. In this

experiment the men were divided into 3 pairs. All 6 were fed the basic formula for 12 weeks, but 2 men serving as controls received pantothenic acid. The other 4 men were deprived of this vitmain, and 2 of these were given an antimetabolite of pantothenic acid.

Thyroid function tests were performed to ascertain any possible aberrations from normal, although none were expected. The responses, while surprising, were unrelated to pantothenic acid or its antagonist, since they were similar in all 6 men.

The protein-bound iodine values which initially averaged 5.2 gamma per 100 ml. of blood, began to rise by the fourth week, and averaged 11.8 by the sixth week of this diet. At the same time the uptake of radioactive iodine began to rise. Initially the 24-hour uptake averaged 13 per cent, but this increased steadily to 32 per cent by the end of the study.

Clinically the men remained euthyroid. Their thyroid glands did not enlarge and their temperature, pulse, and body weight remained constant.

It became apparent that here was a phenomenon not readily explained by our concepts of thyroid metabolism. The only other significant biochemical change was a prompt decrease in the serum cholesterol from average values of 215 to 134 mg. per cent. This is a normal response to a diet containing highly unsaturated fats.

One further consideration arose. The formula was assayed for its content of iodine and found to be low (less than 0.2 mg. per day). Iodine deficiency is known to produce goiter and to increase the radioiodine uptake temporarily, but would the protein-bound iodine also rise? We determined the ratio of bound I^{131} in each man and found it to be normal.

We have considered several possible explanations for this effect but none seems satisfactory. Could unsaturated fats alter the transport of iodine? Perhaps our future studies will reveal whether this is fact or artifact.

59. A SURVEY OF THE RELIABILITY OF THE DISK TEST FOR ANTIBIOTIC SENSITIVITY IN NINE TEACHING HOSPITAL LABORATORIES

R. V. Hoffman, M.D. (By Invitation), G. G. Jackson, M.D., and M. P. Turner, M.S. (By Invitation), Chicago, Ill.

Commercial paper disks for determining bacterial sensitivity to antibiotics are in common use. Their results have considerable influence upon physicians and the treatment of patients. Several studies from individual laboratories have supported or challenged the validity of the test. In the present investigation standard strains of five different bacterial genera were tested by the routine disk method in the laboratories of nine teaching hospitals and by two modifications of the tube dilution method. Major discrepancies occurred in the results from different laboratories in up to 25 per cent of the tests and for individual laboratories in the relation of the results to those obtained by the tube dilution method. The disk test usually showed greater sensitivity than the corresponding tube dilution test. The finding of resistance was more reliable. Over-all, 21 per cent of strains reported as sensitive and 53 per cent of those found moderately sensitive by the disk test were not inhibited by 10 μg/ml. while 11 per cent of strains reported as resistant and 31 per cent reported as moderately resistant were susceptible to this concentration or less. There were marked differences in the correlation of the results for different antibiotics. False sensitivity was common with streptomycin, chloramphenicol, and polymyxin,

whereas false resistance was a more common finding with Aureomycin and tetracycline disks. Important differences also were observed among the bacterial species.

The data illustrate the limited reliability of depending upon the results of routine sensitivity tests in the selection of an antibiotic for therapy. The discussion will include the areas of confidence and independability in the clinical interpretation of the tests.

60. A CRITICAL STUDY OF BALL-TYPE EAR PROTECTORS

RAYMOND F. HOLDEN, JR., M.D., AND KENNETH G. HINK, B.S.E.E. (BY INVITATION), WICHITA, KAN.

In comparing the effectiveness of devices used for the protection of workers against industrial and jet engine noise, promising results have been obtained with a modification of an old design (Plessner, 1885). This modified design, which comprises essentially an anodized aluminum sphere with an adjustable nylon retention knob, has been compared under laboratory conditions with two conventional types of insert ear protectors (bullet and V-51R designs) in 12 adults with normal hearing.

Subjects were tested individually in an efficient sound absorbent room measuring approximately 10 by 12 by 7 feet. A psychoacoustic method of testing was used; i.e., each subject's absolute threshold of hearing was determined before and after inserting ear protectors, the difference being the attenuation afforded by the device under test. Attenuations for each type of ear protector were measured in this manner for noise, octave band widths of noise, and tones. Speech reception thresholds in quiet and noise were measured, using recorded spondee words. The subjects received instructions and sound presentations through loudspeakers located within the sound absorbent room, and signaled awareness thresholds to observers in an adjoining control room.

It was apparent from the experimental data that the criteria employed in selecting the subjects (pure tone audiograms and otoscopic examination) did not insure uniformity of threshold responses, which displayed a definite tendency toward random distribution. By using analysis of variance techniques, it was possible to study the effects of the ear protectors independent of the effects due to subject differences. These studies indicated that the order of decreasing effectiveness for the ear protectors was: (1) ball-type, (2) bullet-type, and (3) V-51R type; octave by octave within the 20 to 9,600 c.p.s. range. The same was true for the speech range, 600 to 4,800 c.p.s. for white noise, and for pure tones. Attenuation of speech reception in quiet tended to be somewhat less than attenuation of white noise. Speech reception in noise appeared to be slightly improved and to about the same extent by all the devices tested.

It was concluded that the ball-type ear protectors were more uniformly effective than the other types which were examined.

61. A NEW ERYTHROPOIETIC SUBSTANCE DERIVED FROM LIVER

DANIEL L. HORRIGAN, M.D., CLEVELAND, OHIO

Previous communications have described two patients with hypochromic anemia which was refractory to the usual hematopoietic agents but which responded to the oral administration of liquid extract of liver (Valentine). This anemia was accompanied in both patients by elevation in serum iron levels,

which became normal during response to therapy. The erythrocytes showed marked variation in size, shape, and color saturation. The bone marrow showed erythroid hyperplasia with maturation arrest at an erythroblastic level. The clinical characteristics of this anemia were very similar to those of a recently reported patient with anemia responsive to pyridoxine but not to oral crude liver extract.

A previously unrecognized and as yet unidentified erythropoietic substance, which produces a hematologic response in one of these patients, has been isolated from the crude liver extract. Procedures for the isolation of the active material include alcohol precipitation and ethyl acetate extraction of the crude extract followed by ion exchange and paper chromatographic techniques. Activity in various fractions is demonstrated in the anemic subject by reticulocyte response, rapid erythrocyte regeneration, and decrease in serum iron concentration to normal values. Hematologic relapse in about four months regularly follows cessation of therapy.

From 90 ml. of the crude extract (equivalent to approximately 680 Gm. of unprocessed liver) about 1.0 mg. of the active material is obtained; this amount when given by mouth daily for 10 days produces an optimal hematologic response in the anemic subject. A suboptimal response may follow daily oral doses of as little as 0.25 mg. for 10 days.

Although the nature of the active substance remains unknown, its ultraviolet spectral characteristics and microbiologic activity do not suggest an identity with any known erythropoietic agent, including pyridoxine and its derivatives. Acid hydrolytic studies suggest a polypeptide structure within the molecule.

The morphologic characteristics of the anemia in this patient suggest a metabolic defect in hemoglobin synthesis. Also, the small amount of the active substance required to correct the anemia suggests that it is a cofactor. The nature of this substance may further elucidate metabolic processes involved in hemoglobin synthesis.

62. HYPOGAMMAGLOBULINEMIA AND CHRONIC LYMPHATIC LEUKEMIA

ROBERT P. HUDSON, M.D. (BY INVITATION), AND SLOAN J. WILSON, M.D.,
KANSAS CITY, KAN.

Patients with chronic lymphatic leukemia (CLL) have decreased resistance to infection. Rundles, Coonrad, and Arends (1954) found hypogammaglobulinemia in one of 15 patients with CLL. Jim (1957) found hypogammaglobulinemia in 34 per cent of 50 CLL patients, but made no attempt to correlate the clinical course with this finding. This study was undertaken to determine the incidence of hypogammaglobulinemia in patients with CLL and to correlate this decrease with (1) duration of disease, (2) type and amount of therapy, and (3) incidence and severity of infectious complications.

Forty consecutive patients (Group A) with CLL were studied. All had been followed at the University of Kansas since diagnosis. Nineteen patients with chronic myelogenous leukemia and 7 with polycythemia vera (Group B) were also studied.

Electrophoresis was by filter paper strip using Spinco apparatus and scanning with an Analytrol. Normal gamma globulin by this method is 16.5 per cent with a range of 12.1 to 20.9 per cent. Values less than 6 per cent were termed severe hypogammaglobulinemia.

Twenty-eight or 70 per cent of the CLL patients had hypogammaglobulinemia, the average having 8.2 per cent gamma globulin. Seven fell in the severe range and none had elevated gamma globulin. Of Group B, 3 (11.5 per cent) exhibited hypogammaglobulinemia and 3 had elevated gamma globulin.

The average duration of disease from diagnosis to electrophoretic study in the 28 CLL patients with hypogammaglobulinemia was 40.4 months compared to 21.8 months in the 12 patients with normal gamma globulin levels. Repeat determinations at 4-month intervals in 12 cases were lower than the initial value in every instance.

Treatment included steroids, urethane, TEM, P^{32}, chlorambucil, and x-radiation. In general, the more lympholytic therapy received, the lower the gamma globulin levels obtained. Since amount of treatment was determined largely by disease duration, no valid conclusions could be reached. Three patients with hypogammaglobulinemia had received no treatment.

Infectious complications in CLL requiring specific treatment occurred more frequently in patients with hypogammaglobulinemia.

It is concluded that patients with chronic lymphatic leukemia have an increased incidence of hypogammaglobulinemia which correlates best with duration of disease. No conclusion could be reached as to its relationship to therapy.

Erratum

In the article by Drs. IRWIN J. FOX and EARL H. WOOD entitled "Use of Methylene Blue as an Indicator for Arterial Dilution Curves in the Study of Heart Disease," which appeared in the October issue, pages 598-612, the fifth and sixth lines in the second paragraph on page 598 should be transposed.

The sentence should read as follows: "Evans blue dye (T-1824), the indicator commonly used, has the disadvantage of causing bluish discoloration of the skin and mucous membranes when given in doses exceeding approximately 0.5 mg. per kilogram[10] which, in addition to the cosmetic effect limiting the number of curves which may be performed, interferes with determination of blood oxygen saturation by oximetry and with the clinical evaluation of cyanosis, as, for example, after cardiac operation."